Molecular Diagnosis of Pancreatic Cancer

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Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctorate of Medicine (MD)

The candidate confirms that the work submitted is her own and appropriate credit has been given where reference has been made to the work of others.

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Abbreviations

ARMS	Amplification refractory mutation system
AT	Ataxia telangiectasia
CEA	Carcino embryonic antigen
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CT	Computed tomography
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ERCP	Endoscopic retrograde colangiopancreatogram
EUROPAC	European Registry
EUS	Endoscopic ultrasound
EUS-FNAB	EUS- fine needle aspiration biopsy
FAMMM	Familial atypical multiple mole melanoma
FAP	Familial adenomatous polyposis
FEPC	Familial excess pancreatic cancer
FISH	Fluorisence in situ hybridisation
FPC	Familial pancreatic cancer
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HNPCC	Hereditary non-polyposis colorectal cancer
HP	Hereditary Pancreatitis

IHC	Immunohistochemistry
LOH	Loss of hetrozygosity
MGMT	O methyl guanine DNA methyl transferase
MRCP	Magnetic resonance cholangiopancreatogram
MT	mutant
OOCR	Ovarian cancer cluster region
PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PET	Positron emission tomography
PSTI	Pancreatic secretory trypsin inhibitor
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RR	Relative risk
SPINK1	Kazal type 1 serine protease inhibitor
SSCP	Single strand conformational polymorphism
TP:FP	True positive: false positive
UICC	Union Internationale Contre le Cancer
USS	Ultrasound

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I am grateful to all the individuals who have given me advice and assistance to enable me to complete this thesis. In particular, I would like to thank Professor J P Neoptolemos for giving me the opportunity to do an MD project in his department and for the constant encouragement and support during my time as a researcher and in my clinical career. Thank you.

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Vitone LJ, Greenhalf W, Howes N, **Wong T**, Mountford R, Blake V, Neoptolemos JP.

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Dedication

This thesis is dedicated to Ali and our children

Laura and Alex

Abstract

The prevalence of pancreatic cancer in the general population is too low--even in high prevalence areas such as Northern Europe and North America (8-12 per 10^5 population)--relative to the diagnostic accuracy of present detection methods to permit primary screening in the asymptomatic adult population. The lifetime risk of developing pancreatic cancer for patients with hereditary pancreatitis is 20% by the age of 60 years and 40% by the age of 70 years, an aim of this thesis is to establish if this risk can be further stratified according to genotype or founder group, despite further clarifying the role of founder effect no significant stratification by genotype was possible. A 40% lifetime risk is still low given current screening modalities. Conventional radiological imaging methods such as endoluminal ultrasound and endoscopic retrograde pancreatography have proved valuable in the detection of early neoplastic lesions in patients with familial pancreatic cancer, but these are less effective in individuals with gross morphological features of chronic pancreatitis as seen in most cases of HP. Detection of molecular changes in early or pre-malignant lesions could help in distinguishing pancreatitis and cancer. In this thesis I will show that although mutant K-ras is common in the pancreatic juice of patients with pancreatic cancer, it is also present in patients with chronic pancreatitis, as well as increasingly in the older population without pancreatic disease. Nevertheless, the presence of mutant K-ras may identify a genuinely higher-risk group, enabling additional diagnostic imaging and molecular resources to be focussed on such a group. I have also shown that epigenetic changes (promoter methylation) detected in pancreatic juice can be used to further stratify risk and to clarify the relationship of molecular changes with age.

Chapter 1- Pancreatic Cancer

Incidence and Mortality

Pancreatic ductal adenocarcinoma, PDAC, is one of the top ten causes of cancer-related death in the Western world (Fernandez et al. 1994; Bramhall et al. 1995). Although the prevalence of pancreatic cancer in Northern Europe and North America is not especially high (8-12 per 10^5 population) the disease is virtually universally fatal (Gudjonsson 1995; Sener et al. 1999).

The incidence of pancreatic cancer has reached a plateau in recent years in England and Wales. The incidence in males started falling in the early 1980s. However, in females, there was a gradual increase in the 1970s and 1980s before starting to decline in the 1990s as shown in Figure 1 (Quinn et al. 2006). The incidence of pancreatic cancer is approximately 7-10 per 100,000 populations with a slight preponderance towards males.

By comparison, in the United States, the incidence of pancreatic cancer has increased three-fold from 1920- 1978, possibly due to improved diagnostic techniques, but has remained relatively constant since. The incidence is approximately 10-12 per 100,000 populations, again with a higher incidence in males.

The sharp increase in incidence of this malignancy was also noted in Japan- this country now has an incidence rate for pancreatic cancer comparable with its western counterparts.

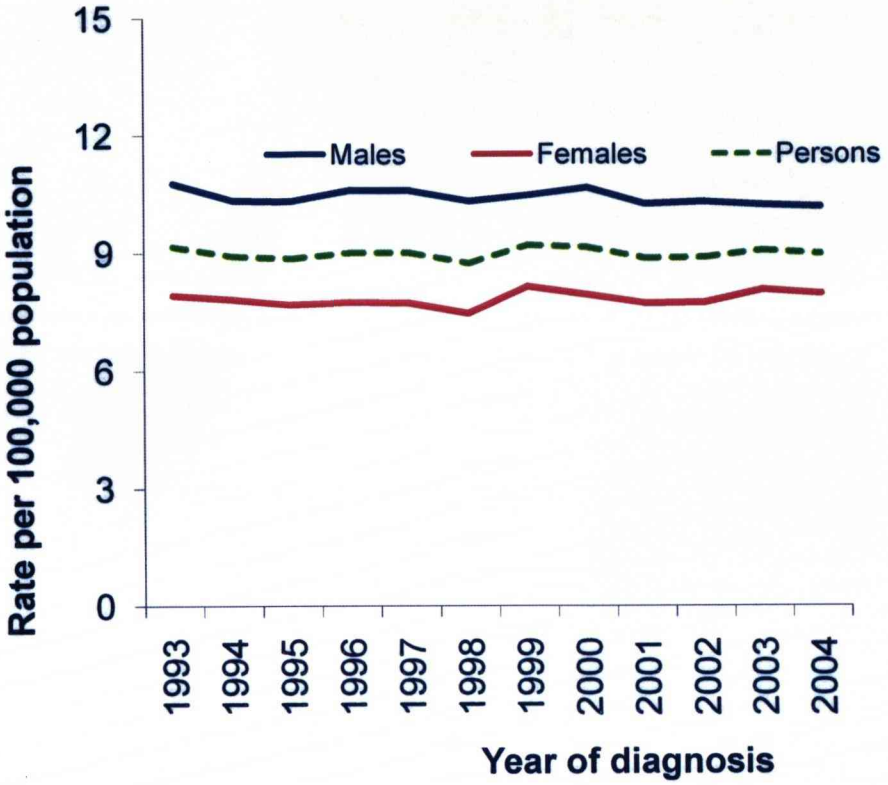


Figure 1: Age standardised (European) incidence rates, pancreatic cancer, by sex, UK, 1993-2004 (Quinn et al. 2006)

(Taken from http://info.cancerresearchuk.org/cancerstats/types/pancreas/ without permission)

The diagnosis of pancreatic cancer bears grim consequences to a patient- imminent death usually within 12 months in 90% of cases. Pancreatic cancer is a malignancy with an aggressive nature and ranks amongst the top ten most common cause of cancer death in the western world.In the 1999 report from the Cancer Research Campaign, there were 6770 new cases of pancreatic cancer diagnosed in England and Wales in 1996. In 1998, 6560 deaths were attributed to this cancer (Coleman et al. 1999), accounting for an annual mortality of ~3% of cancer deaths in the United Kingdom. In the United States and Europe, PDAC accounts for 28000 and 40000 deaths per year respectively.

Pancreatic cancer poses a great diagnostic problem to clinicians worldwide. Patients remain well for a long time before symptoms arise. In the majority of cases, when the disease is clinically evident, more than 80% of patients have positive regional lymph node or distant metastasis. The median survival of these patients is 4-6 months with few, if any, survivors after five years. Only a small proportion of these patients (5-10%) will be suitable for surgical resection and even with surgery the five-year survival rate is 10-24% (Allema et al. 1995; Nitecki et al. 1995; Yeo et al. 1995; Pedrazzoli et al. 1998) but there may be a small to modest improvement with adjuvant treatment (Ghaneh et al. 1999). Even though respectable five-year survival figures may be achieved with resection and adjuvant treatment, virtually all of these patients are also dead within the next 2-3 years. Very early cancers (<1cm in diameter and no lymph node metastases) may result in five-year survival rates of ~50% and the prospect of genuine cure – unfortunately such tumours are rarely found in routine clinical practice (Tsuchiya et al. 1986).

Despite advances in techniques for resectional surgery, safer anaesthesia for more radical operative procedures, trials for adjuvant treatment regimes and modern diagnostic modalities, the overall 5 year survival outlook has remained poor.

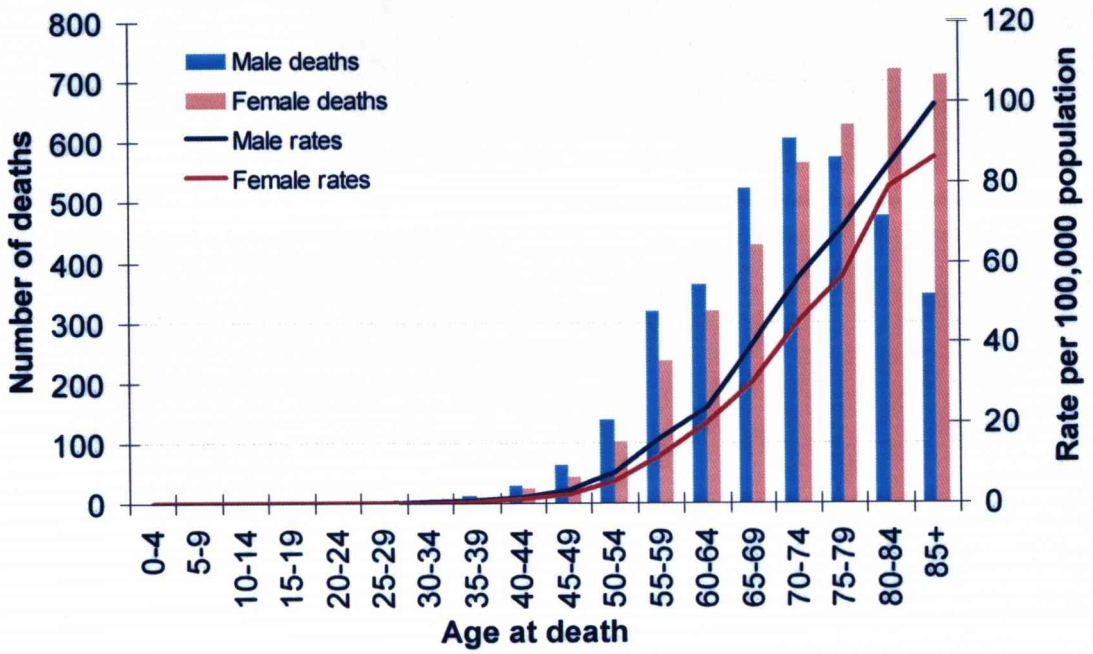


Figure 2: Number of deaths and age-specific mortality rates, pancreatic cancer, by sex, UK, 2005 (Quinn et al. 2006)

(Taken from http://info.cancerresearchuk.org/cancerstats/types/pancreas/ without permission)

Each year, approximately 7000 deaths are attributed to pancreatic cancer in the United Kingdom, 40,000 in Europe and 28,000 in the United States. The median survival of these patients is 4-6 months with few, if any, survivors after five years. Only a small proportion of these patients (5-10%) will be suitable for surgical resection and even with surgery the five-year survival rate is 10-24% (Allema et al. 1995; Nitecki et al. 1995; Yeo et al. 1995; Pedrazzoli et al. 1998) but there may be a small to modest improvement with adjuvant treatment (Ghaneh et al. 1999). Although respectable five-year survival figures may be achieved with resection and adjuvant treatment, virtually all of these patients are also dead within the next 2-3 years. Survival from this malignancy declines with age.

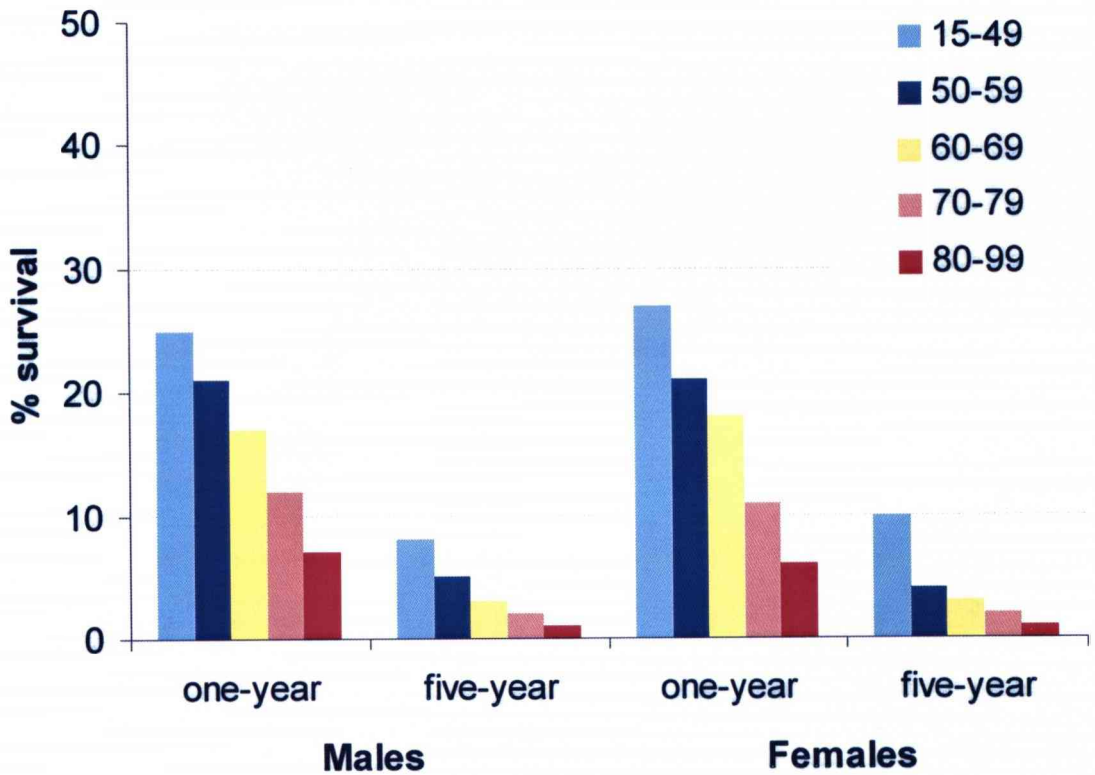


Figure 3: One- and five-year relative survival by age and sex, patients diagnosed with pancreatic cancer, England, 1998-2001 and followed up to the end of 2003 (Quinn et al. 2006)

(Taken from http://info.cancerresearchuk.org/cancerstats/types/pancreas/ without permission)

Definitive Risk Factors

Age

Pancreatic cancer is extremely rare under the age of 40 and from then, the risk increases approximately linearly Figure 4.

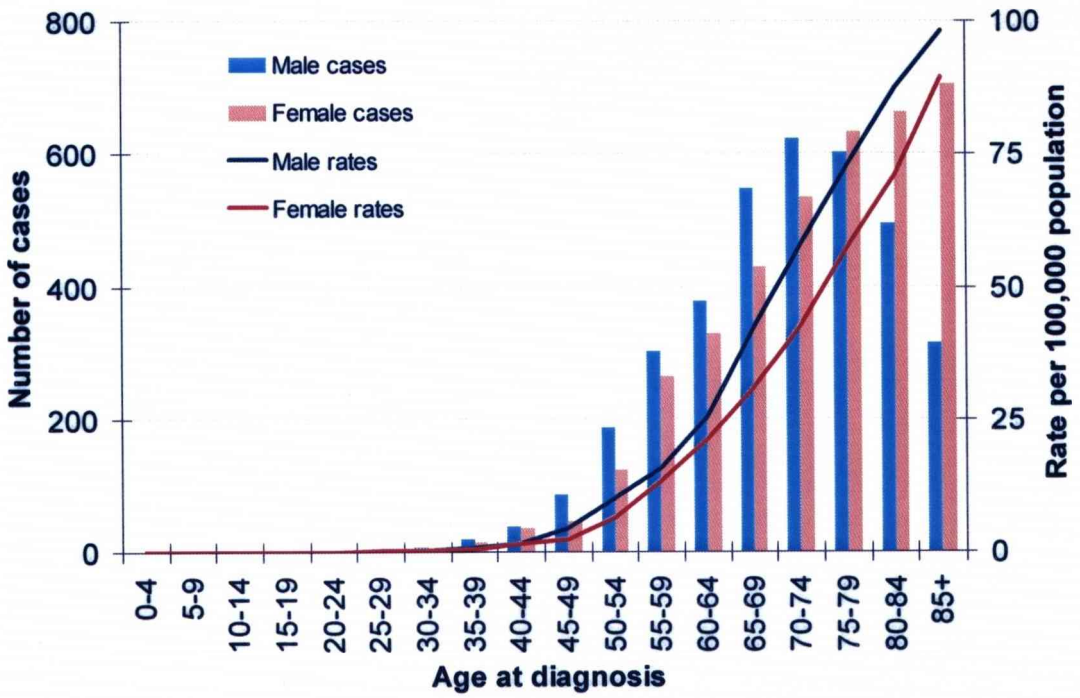


Figure 4: Numbers of new cases and age specific incidence rates, by sex, pancreatic cancer, UK 2004 (Quinn et al. 2006)

(Taken from http://info.cancerresearchuk.org/cancerstats/types/pancreas/ without permission)

Race

The age-adjusted incidence and death rates from pancreatic cancer vary from country to country- the incidence in India and Singapore is markedly lower than England and Wales, the United States and Japan (Quinn et al. 2006). There is also ethnic group variability- in the United States, the incidence rates were highest in African American when compared with Caucasians or Asian Americans.

Smoking

This appears to be the most consistent risk factor associated with pancreatic cancer. The mortality ratio from pancreatic cancer in smokers compared to non-smokers has been shown to be around 2:1 in the United States, UK and Japan (Hirayama 1989). A population-based, case-control study of 526 cigarette smokers and 2153 control subjects by Silverman et al (Silverman et al. 1994) showed a 70% increased risk of pancreas cancer in the former group compared with the later group. The overall proportion of pancreas cancer attributable to cigarette smoking was 29% in blacks and 26% in whites. The link between smoking and pancreas cancer were supported by further studies by Siemiatycki et al (Siemiatycki et al. 1995), Fernandz et al (Fernandez et al. 1996), and Partanen et al (Partanen et al. 1997) - all these authors reported attributable risk percentages ranging between 14-33.

The influence of smoking cessation was looked at by Fuchs and they found a 48% reduction in pancreatic cancer risk within two years of smoking cessation and the risk was reduced to that of a non-smoker after ten years (Fuchs et al. 1996).

The risks of pancreatic cancer from cigar smoking was analysed by Shapiro et al (Shapiro et al. 2000) – although cigar smoking did not appear to increase the risk of pancreatic cancer related mortality (relative risk 1.3, 95% CI 0.9-1.9), if a smoker inhaled the smoke, there was an increased risk of developing pancreatic cancer (relative risk 2.7, 95% CI 1.5-4.8) (Shapiro et al. 2000).

Pancreatic Cancer in High Risk Groups

The prevalence of pancreatic cancer in the general population is too low - even in high prevalence areas such as Northern Europe and Northern America (8-12 per 10^5 population) (Gudjonsson 1995; Sener et al. 1999) - relative to the diagnostic accuracy of present detection methods to permit primary screening in the asymptomatic adult population.

The recognition that the lifetime risk of developing pancreatic cancer in high risk groups such as patients with Hereditary Pancreatitis (HP) is extremely high (20% by the age of 60 years and 40% by the age of 70 years) (Howes et al. 2002) poses considerable challenges and opportunities for secondary screening in those patients without any clinical features of pancreatic cancer. Even for secondary screening, the requirements for cancer detection at a biological stage that would be amenable to cure by surgery (total pancreatectomy) still require diagnostic modalities with a very high sensitivity and specificity. Conventional radiological imaging methods such as endoscopic ultrasound (EUS) and endoscopic retrograde cholangiopancreatogtram (ERCP), which have proved to be valuable in the early detection of early neoplastic lesions in patients with Familial

Pancreatic Cancer (FPC), may well be applicable to patients with HP but only in those without gross morphological features of chronic pancreatitis (other than parenchymal atrophy). Unfortunately, most cases of HP also have associated gross features of chronic pancreatitis that are likely to seriously undermine the diagnostic value of these conventional imaging modalities (Muller et al. 1994; Midwinter et al. 1999).

High Risk Conditions for Developing Pancreatic Cancer

Pancreatic cancer may develop in conditions with pre-existing pancreatic disease (chronic pancreatitis, HP and cystic fibrosis), as part of a 'pure' Familial Pancreatic Cancer (FPC) syndrome, in families in which there is an excess of pancreatic cancers (FEPC) and as part of a recognized familial cancer syndrome.

Disorder/ Other Associated Neoplasms	Genes Identified Or Implicated	Mode of Involvement	Fold-Risk Of Pancreatic Cancer
Chronic Pancreatitis Increased risk of tobacco-associated cancers: laryngeal cancer; lung cancer; oesophageal cancer.	Possible disease modifying genes: SPINK1; CFTR; others	Polymorphic genetic factors and environmental factors (alcohol).	5-15
Hereditary Pancreatitis (HP)	PRSS1 (in ~70%)	Autosomal dominant; point mutations; ~80% penetrance.	70-100
Cystic Fibrosis (CF)	CFTR	Autosomal recessive; point mutations (both alleles); mutations in 5% of the population.	~5
Familial Pancreatic Cancer (FPC)	Unknown	Autosomal dominant; > 100% penetrance.	Unknown
Families with Excess Pancreatic Cancers (FEPC)	Unknown	Unknown.	Unknown
Familial Atypical Multiple Mole Melanoma (FAMMM) Melanomas; GI tract cancers; gynaecological cancers; respiratory tract cancers; and renal system tumours.	CDKN2A CDK4	Autosomal dominant; point mutations.	13-65
Puetz-Jeghers Syndrome GI tract cancer, breast cancer, uterine cancer, ovarian cancer, lung cancer	STK11/ LKB1	Autosomal dominant; splice site and intragenic sequence mutations.	Up to 132
Familial Breast Cancer Ovarian cancer <50 years; stomach cancer; leukaemia; lymphoma.	BRCA1	Autosomal dominant; > 100% penetrance; point mutations; in Ashkenazi Jews, 1.1% have the 185delAG and 0.13% the 5382insC mutations.	Unknown
Familial Breast Cancer Ovarian cancer >60 years; male breast cancer; melanoma; gastric cancer; prostate cancer; colorectal cancer	BRACA2	Autosomal dominant; > 100% penetrance; point mutations; 6174delT mutation in 1.4% of Ashkenazi Jews.	3.5-10
Ataxia Telangiectasia Haematological malignancies; breast cancer.	ATM	Autosomal recessive; point mutations; mutations in 0.2-1% of the population.	Unknown
Hereditary Non-Polyposis Colon Cancer Syndrome (HNPCC – Lynch II) Colon cancer; gastric cancer; small bowel cancer; endometrial cancer; ovarian cancer; urothelial tumours.	*h*MLH1; *h*MSH2, *h*MSH6; *hP*MS1, *hP*MS2	Autosomal dominant; point mutations.	Unknown
Familial Adenomatous Polyposis (FAP) Colorectal cancers; small bowel tumours; thyroid cancers; desmoid tumours.	APC (in ~80%)	Autosomal dominant; point mutations; genomic rearrangements.	4.5
Li-Fraumeni Sarcomas; breast cancer; brain tumours; lung cancer; leukaemias.	TP53 (in ~50%)	Autosomal dominant; point mutations.	Unknown

Table 1: Conditions with increased susceptibility for PDAC

Chronic Pancreatitis

Around 70% of cases of chronic pancreatitis are related to chronic alcohol abuse and only a tiny proportion has an obvious environmental, metabolic or anatomical cause. Tropical pancreatitis has been postulated to be one end of the spectrum of presentation for chronic pancreatitis. The patients with tropical pancreatitis are usually from India, Africa and Asia. The patients are mostly young (average age of onset 12-15 years), there is a high incidence in females, the malnourished and the disease shows rapid progression and causes severe pancreatic damage with multiple large ductal calculi. There is usually no history of alcoholism or biliary tract disease (Balakrishnan et al. 2006). The SPINK1 N34S gene mutation has been show to play a role in the increased risk of tropical pancreatitis development (Rossi et al. 2001; Schneider et al. 2002).

Many of the remainder may be related to the inheritance of particular gene variants that may modify the disease such as mutation of the CF gene (CFTR) (Cohn et al. 1998) and SPINK1 in Hereditary Pancreatitis (Pfutzer et al. 2000; Witt et al. 2000).

The diagnosis of chronic pancreatits is based on the fulfilment of one or more of the following criteria: (i) pancreatic calcifications on x ray, computed tomography (CT) scan, ultrasonography (USS), or EUS (hyperechoic foci, hyperechoic strands, lobularity, cysts, calcifications, duct dilation, duct irregularity, hyperechoic duct margins, visible side branches, and intraductal calcifications) (ii) moderate to marked pancreatic ductal lesions on endoscopic retrograde or intraoperative pancreatography ("Cambridge" criteria); (iii) typical histology on an adequate surgical pancreatic specimen.

There is wide variation in the estimation of the increased risk of pancreatic cancer from chronic pancreatitis, ranging from to 2.3 to 18.5-fold (Bansa et al. 1995; Talamini et al. 1999). Lowenfels et al with the International Pancreatitis Study Group showed that the 10 and 20 year cumulative incidence of developing pancreatic cancer after a diagnosis of chronic pancreatitis was 1.8% and 4% respectively (Lowenfels et al. 1993). This was equivalent to a 15- to 16-fold increased risk compared to the general population. One Italian case-control study estimated that the risk of developing pancreatic cancer after follow-up for at least five years of cases with chronic pancreatitis was 5-fold (Fernandez et al. 1995) whilst the risk in another study from the same country was found to be 13-fold (Talamini et al. 1999; Talamini et al. 2000). Studies from Sweden, however, reported only a marginal two-fold risk after 10 years follow up, bringing into question whether chronic pancreatitis was a genuine risk factor in addition to other confounding factors, in particular tobacco consumption (Ekbom et al. 1994; Karlson et al. 1997).

Howes et al highlighted that in a study by Malka et al involving mainly chronic pancreatitis patients (85% of the 373 patients), the overall increased risk of pancreatic cancer was significant with a standardised incidence ratio 26.7 (Howes et al. 2002; Malka et al. 2002).

Hereditary Pancreatitis

HP is a rare autosomal dominant condition (with ~80% penetrance) characterized by the early onset of acute pancreatitis that progress to chronic pancreatitis. In around 70% of cases, the cause is one of several mutations in the cationic trypsinogen gene (PRSS1) and in the remainder of cases the gene(s) responsible is (are) not known (Whitcomb et al. 1996; Gorry et al. 1997). In the table below (Table 2), is a list of the database of PRSS1, PRSS2 and SPINK1 variants for HP as published by the University of Leipzig (http://www.uni-leipzig.de/pancreasmutation/db.html March 2008).

Table 2: Mutations in pancreatitis adapted from the University of Leipzig database

gene	amino acid variant	location	nucleotide change	patients
PRSS1		5'UTR3	-28delTCC	1
PRSS1		intron 1	IVS1+40delC	1
PRSS1	p.A16V	exon 2	c.47C>T	>10
PRSS1	p.D19A	exon 2	c.56A>C	1
PRSS1	p.D22G	exon 2	c.65A>G	1
PRSS1	p.K23R	exon 2	c.68A>G	1
PRSS1	p.N29I+N54S	exon 2	large conversion from PRSS2: C.41-34_c.200+236conNM_002770.2:c.41-34_c.200+236	1
PRSS1	p.N29I	exon 2	c.86A>T	>50
PRSS1	p.N29T	exon 2	c.86A>C	1
PRSS1	p.P36R	exon 2	c.107C>G	1
PRSS1	p.Y37X	exon 2	c.111C>A	1
PRSS1	p.V39A	exon 2	c.116T>C	1
PRSS1		exon 2	IVS2+1G>A	1
PRSS1	p.E79K	exon 3	c.235G>A	4
PRSS1	p.G83E	exon 3	c.248G>A	1
PRSS1	pI88N	exon 3	c.263T>A	1
PRSS1	p.K92N	exon 3	c.276G>T	1
PRSS1	p.D100H	exon 3	c.298G>C	1
PRSS1	p.L104P	exon 3	c.311T>C	1
PRSS1	p.R116C	exon 3	c.346C>T	3
PRSS1	p.R122C	exon 3	c.364C>T	2
PRSS1	p.R122H	exon 3	c.365G>A	>100
PRSS1	p.R122H	exon 3	c.365G>A, c.366C>T	2
PRSS1	p.V123M	exon 3	c.367G>A	1
PRSS1	p.T137M	exon 3	c.137C>T	1
PRSS1	p.C139S	exon 3	c.415T>A	1
PRSS1	p.C139F	exon 3	c.416G>T	1
PRSS1		intron 4	IVS4-8C>T; IVS4-11C>T	1
PRSS1		intron 4	IVS4-24C>T	3
PRSS1	p.D162D	exon 4	c.486G>T	polymorphism
PRSS1	p.G208A	exon 5		1
PRSS1	p.N246N	exon 5	c.738C>T	polymorphism
PRSS2	p.G191R	exon 4	c.738C>T	c.571g>a

gene	amino acid variant	location	nucleotide change	families
SPINK 1	large deletion	promotor - intron 2	c.1–320_c.55+961del1336 bp	1
SPINK 1		promotor	c.1-253T>C	polymorphism
SPINK 1		promotor	c.1-215G>A	1
SPINK 1		promotor	c.1-215G>T	1
SPINK 1		promotor	c.1-164G>C	polymorphism
SPINK 1		promotor	c.1-147A>G	2
SPINK 1		promotor	c.1-142T>C	1
SPINK 1		5' UTR	c.1-53C>T	1
SPINK 1		promotor	c.1-41G>A	5
SPINK 1		promotor	c.1-7T>G	polymorphism
SPINK 1	p.M1T	exon 1	c.2T>C	1
SPINK 1	p.L12F	exon 1		4
SPINK 1		exon 1	c.27delC	2
SPINK 1	L14P	exon 1	41T>C	1
SPINK 1		intron 1	IVS1-62T>C	polymorphism
SPINK 1		intron 1	IVS1-37T>C	linkage with N34S
SPINK 1	S25S	exon2	75C>T	polymorphism
SPINK 1		intron 2	IVS2+268A>G	linkage with N34S
SPINK 1		intron 2	IVS2-352A>G	polymorphism
SPINK 1		intron 2	c.87+1G>A	1
SPINK 1	Y33stop	exon 3	98insA	polymorphism
SPINK 1	p.N34S	exon 3	c.101A>G	>200
SPINK 1	p.D50E	exon3	c.150T>G	1
SPINK 1	p.Y54H	exon3	c.160T>C	1
SPINK 1	p.P55S	exon 3	c.163C>T	<10
SPINK 1	p.R65Q	exon 3	c.194G>A	1

gene	amino acid variant	location	nucleotide change	families
SPINK 1	p.K66N	exon 3		1
SPINK 1		intron 2	IVS2-23A>T	polymorphism
SPINK 1		intron 3	IVS3+2T>C	<10
SPINK 1		intron 3	IVS3-1643G>C	polymorphism
SPINK 1		intron 3	IVS3-604G>A	linkage with N34S
SPINK 1		intron 3	IVS3-476T>G	polymorphism
SPINK 1		intron 3	IVS3-321C>T	polymorphism
SPINK 1		intron 3	IVS3-66-65insTTTT	linkage with N34S
SPINK 1		intron 3	IVS3+125C>A	1
SPINK 1		intron 3	IVS3+184T>A	<10
SPINK 1	p.R67C	exon 4	c.199C>T	1
SPINK 1	p.G77G	exon 4	c.231G>A	polymorphism
SPINK 1		3'UTR	272C>T	polymorphism

In seminal study by Lowenfels et al and the International Pancreatitis Study Group, they estimated that the cumulative lifetime risk (to the age of 70 years) for pancreatic cancer was ~40% in patients with HP (Lowenfels et al. 1993; Lowenfels et al. 1997). This has been confirmed in a larger study by Howes et al and collaborators in the European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) who found that lifetime risk of developing pancreatic cancer was also 60% (95% confidence intervals [CI], 45-75%) (Howes et al. 2000).

Lowenfels et al also reported that patients with a paternal transmission of HP was associated with a much greater increased lifetime risk of developing pancreatic cancer (~70%) (Lowenfels et al. 1997; Lowenfels et al. 1999), but the EUROPAC data clearly showed that the risk is identical (~40%) irrespective of the parental gender-mode of transmission (Lerch et al. 1999; Howes et al. 2004).

The EUROPAC data demonstrated that the risk of pancreatic cancer was extremely low in patients with HP aged <40 years but rose from this age such that the risk was 20% by the age of 60 years and 40% by the age of 70 years (Howes et al. 2002). The level of risk was further confirmed in a study by the EUROPAC group in 2004, with the cumulative risk of pancreatic cancer estimated at 44% at 70 years from symptom onset with a standardized incidence ratio of 67% Figure 5 (Howes et al. 2004).

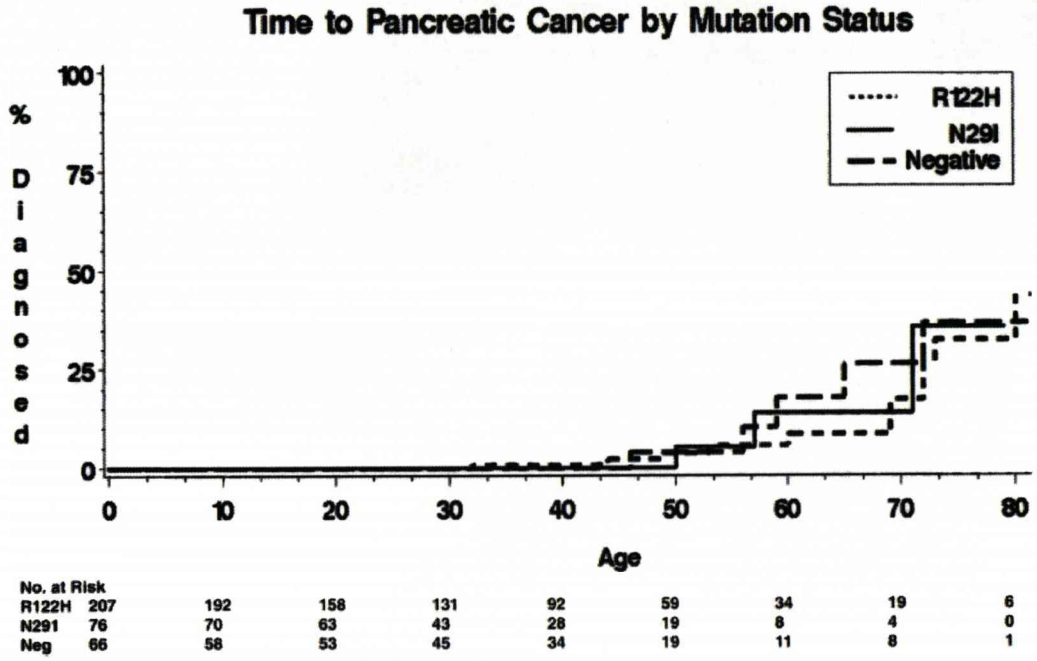


Figure 5: The time to pancreatic cancer in patients with Hereditary Pancreatits (Howes et al. 2004).

This shows that there are no significant differences by mutation status and that lifetime risk is approximately 40% (to age 75).

Despite all the advances in our understanding of the pathobiology of pancreatic cancer and the diagnostic technology available, the mortality rate of this disease is still closely mirrored by the incidence. Pancreatic cancer develops as a result of interplay between genetic and environmental factors. With intensive studies of the epidemiology and genetic nature of this disease, hopefully, one day soon we will be able to fully understand the exact nature of pancreatic cancer and work towards further improving the dismal survival figures.

Cystic Fibrosis

CF is the commonest known genetic disease that is inherited in an autosomal recessive manner. The CF gene encodes the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein, a membrane-bound chloride channel. In CF the function of CFTR is impaired or altogether lost due to one of around 1,000 point mutations in each of the two inherited alleles. The damage to the pancreas in cystic fibrosis begins in utero. Due to the previous limited life expectancy of patients with CF, the risk of pancreatic cancer was not certain and indeed hardly relevant. But now patients with CF are living longer and longer due to great improvements in medical care.

In 1993, a British study of 412 patients with CF showed a significantly increased observed to expected ratio of pancreatic cancers– but this estimate was based on only two observed pancreatic cancers (Sheldon et al. 1993). A study in 1995 based on 28,511 patients with CF from Canada and the USA confirmed the increased risk for pancreatic cancer and all digestive tumours but the overall cancer risk was not elevated (Neglia et al. 1995). In 1994, a case of pancreatic cancer was reported (the fifth case up to then) in a 39-year-old man with CF carrying the common ΔF508 mutation in the CFTR gene (Tsongalis et al. 1994).

Maisonneuve et al, in a study published in 2003, concluded that in adult CF patients, there is an increased risk of digestive tract tumours, especially small bowel, colon and biliary tract tumours (Maisonneuve et al. 2003). The increased risk was more pronounced in patients who had had organ transplantation. In a follow up report published in 2007, the group reaffirmed that although the estimated risk of pancreatic

cancer in CF is five to six times greater than in the general population, the absolute risk of pancreatic cancer in patients with CF is negliglible (Maisonneuve et al. 2007).

Family History

The appreciation that aggregation of pancreatic cancer occurs in certain families has emerged over the past 15 years or so. A case control study from Louisiana, reported a 5.35% (95% CI, 2.1-13.2%) relative risk of pancreatic cancer among relatives with the same cancer. The relationship between family history and the risk of pancreatic cancer was specifically investigated in first-degree relatives in a case-control study conducted in Northern Italy. The relative risk was found to be 3.0 (95% CI, 1.4-6.6) in first-degree relatives compared to control. This level of relative risk was confirmed in a study on the Francophone community from Canada, in which 7.8% of patients with pancreatic cancer were reported to have a first-degree relative with the same disease, a 13-fold difference compared with matched control cases.

An analysis from The John's Hopkins registry (USA) found that 3.7% of second-degree relatives developed pancreatic cancer compared with 0.6% in cases without a family history. In the latest investigation, a population-based case-control study of pancreatic cancer was conducted in three areas of the USA (Silverman et al. 1999). This study found a significantly increased risk for pancreatic cancer in subjects reporting first-degree relatives not only for pancreatic cancer (odds ratio [OR] =3.2) but also for colon cancer (OR=1.7) or ovarian cancer (OR=5.3). There was a non-significant elevated risk for cancers of the endometrium (OR=1.5) or breast (OR=1.3).

Familial Pancreatic Cancer

FPC is a rare family cancer syndrome that is poorly understood and with an apparent autosomal dominant mode of inheritance with restricted but unknown level of penetrance (Lynch et al. 1996). Much more common than FPC is the aggregation of pancreatic cancer within certain families, either as the only cancer type or in association with other cancers (Lal et al. 2000).

Although there is a probable inheritance pattern in patients with FPC, the causative gene has yet to be identified. However, although patients with Puetz-Jeghers syndrome is associated with a ~100 fold increased risk of pancreatic cancer development, there is strong evidence from Grützmann et al that the LKB1/STK11 gene inactivation does not play a part in the genetics of FPC (Grutzmann et al. 2004).

Familial Atypical Mole-Multiple Melanoma

FAMMM is an autosomal dominant disease associated with multiple atypical naevi, malignant melanoma and extra-cutaneous malignancies. Germ-line mutations of the CDKN2A gene (that encodes $p16^{INK4a}$) have been found to co-segregate with melanoma in a sub-set of FAMMM kindreds with linkage to the CDKN2A locus on chromosome 9p21 which occur in about 25% of all FAMMM families (Hussussian et al. 1994; Kamb et al. 1994).

CDKN2A is an important tumour suppressor gene and is functionally altered in ~85% cases of sporadic pancreatic cancers by a variety of genetic mechanisms (Caldas et al. 1994). The $p16^{INK4a}$ protein is critically involved in cell cycle control by binding to cyclin-dependent kinases -4 (cdk4) and -6 (cdk6) inhibiting the formation of catalytic complexes with cyclin D and hence the phosphorylation of pRb, which is essential for cell cycle progression through G_1 (Serrano et al. 1993; Fang et al. 1998). Indeed other families with individuals with multiple melanomas may also have germline mutations of the cdk4 (CDK4) gene on chromosome 12q13 (Zuo et al. 1996).

A Dutch study of 200 individuals from nine FAMMM families reported that nine of 43 cancers identified were pancreatic producing an observed to expected frequency ratio of 13.4 (Bergman et al. 1990). A USA study by Goldstein et al found a 22-fold increased risk of pancreatic cancer (7 cases observed vs 0.32 expected; standardized incidence ratio, 21.8; 95% CI, 8.7- 44.8) in FAMMM patients with functionally impaired CDKN2A mutations when compared to patients with functionally intact CDKN2A (Goldstein et al. 1995). Interestingly, there are families with and without pancreatic cancer that have the same CDKN2A mutations, indicating additional mechanisms to the development of pancreatic cancer in these families (Goldstein et al. 2000).

A study by Vasen et al specifically investigated 27 Dutch FAMMM families for the p16-Leiden mutation (a 19 bp deletion in exon 2 of the CDKN2A gene). They found the p16-Leiden mutation in 19 families that included 86 patients with melanoma. The second most frequent cancer was pancreatic cancer, which was observed in 15 patients from

seven families at a mean age at diagnosis of 58 years (range 38-77 years). The estimated cumulative risk of developing pancreatic cancer in putative mutation carriers by the age of 75 years was 17% (compared to 0.5-0.9% for the general population).

In Germany, Bartsch et al analysed five families with a pancreatic cancer-melanoma syndrome (families with at least one patient with histologically confirmed pancreatic cancer and at least one first-degree relative with histologically confirmed malignant melanoma) for the $p16^{INK4a}$ mutations and found two of the five families to carry a germline mutation (Bartsch et al. 2002). This group observed that the age of occurance of the pancreatic cancer/ malignant melanoma were about 15 years younger than in the general German population. Bartsch et al speculated that the phenotypic prevalence of pancreatic cancer and melanoma among $p16^{INK4a}$ mutation carriers might be explained by an early inactivation of $p16^{INK4a}$ together with modifying genes, partly or completely suppressing the pancreatic cancer or melanoma phenotype.

Peutz-Jeghers Syndrome

Peutz-Jeghers syndrome is an autosomal dominant disease characterized by the presence of hamartomatous polyps in the gastrointestinal tract, muco-cutaneous melanin pigmentation and various gastrointestinal and non-digestive tract cancers. The gene responsible is LKB1/STK11 (located on chromosome 19p13), a tumour suppressor gene that encodes for a serine-threonine kinase and is postulated to inhibit cell proliferation at the level of G1 (Hemminki et al. 1998; Jenne et al. 1998). Studies have indicated a significantly increased risk of pancreatic cancer, other digestive tract cancers including

gastric and small bowel and also breast, ovarian and endometrial cancers with an overall 10 to 20-fold risk (Giardiello et al. 1987; Hizawa et al. 1993; Boardman et al. 1998). Giardiello et al recently performed an individual patient meta-analysis to determine the risk of cancer risk in 210 patients with Peutz-Jeghers syndrome (Giardiello et al. 2000). The relative risk (RR) for all cancers was 15.2 (95% CI, 2-19%) with a cumulative risk for all cancers of 93% between the ages of 15 and 64 years. There was a statistically significant increase of the RR for pancreatic cancer (132; 95% CI, 44-261%).

Familial Breast Cancer And Breast /Ovarian Cancer Syndromes (BRCA1 and BRCA2)

Mutations of the BRCA1 and BRCA2 genes account for around 5-10% of all breast cancers (Iau et al. 2001) and in one Canadian study accounted for 11.7% (95% CI, 9.2%-14.8%) of all ovarian cancers. Pancreatic cancer as well as ovarian, colorectal, stomach and prostate cancers occurred among first-degree relatives of carriers of BRCA2 mutations only when mutations were in the ovarian cancer-cluster region (OCCR) of exon 11, whereas an excess of breast cancer was seen when mutations were outside the OCCR, The penetrance of BRCA2 mutations for cancers of all sites combined was greater for men (53%) than for women (38%) (Risch et al. 2001).

The prevalence of BRCA1 and BRCA2 mutations in the general population is high and estimates vary from 0.12% to 0.66% (Iau et al. 2001) but is even higher in Ashkenazi Jews: the 185delAG BRCA1 mutation occurs in 1.1%, the 5382insC BRCA1 mutation is found in 0.13% and the 6174delT BRCA2 mutation occurs in 1.4% (Roa et al. 1996).

Indeed it has been estimated that the prevalence of BRCA1 and BRCA2 mutations in Ashkenazi Jews may be even higher (2.0-2.5%) (Warner et al. 1999).

Tonin et al found a frequency of 4.1% BRCA2 mutations amongst 220 Jewish families affected by breast cancer. Pancreatic cancer was present in 19 of these families and was predictive of the presence of a germline mutation. There was a BRCA1 or BRCA2 mutation in 14/19 families (74%; 11 in BRCA1 and 3 in BRCA2) whilst in the absence of pancreatic cancer there was no mutation in either gene in 53% of families (Tonin et al. 1996). Thorlacius et al identified the 999del5 BRCA2 mutation in 16 out of 21 Icelandic families with male and female breast cancer, prostate cancer and ovarian cancer. There were 11 pancreas cancers in the mutation-positive families but none in mutation-negative families (Thorlacius et al. 1996).

Phelan et al reported similar findings in a study of 49 families with site-specific breast cancer eight of which had a BRCA2 mutation. Pancreatic cancer occurred in 4/8 mutation-positive families compared to only 5/41 mutation-negative families and occurred at a younger age in those with a mutation (Phelan et al. 1996). A similar pattern of disease association between pancreatic cancer and the germline mutation occurs in the case of BRCA1 breast cancer families. A study by Johannsson et al of 47 kindreds from southern Sweden identified a BRCA1 germline mutation in 15 kindreds and another with linkage to the BRCA1 region. There were two cases of pancreatic cancer (diagnosed at 42 and 54 years of age) both of whom had inherited the at-risk haplotype (Johannsson et al. 1996).

The Breast Cancer Linkage Consortium investigated 173 breast-ovarian cancer families with BRCA2 mutations from 20 centres in Europe and North America and observed a significantly increased RR for pancreatic cancer (3.51; 95% CI, 1. 87-6.58%). (The Breast Cancer Linkage Consortium 1999).

An alternative way of investigation is to study the prevalence of germline mutations in patients with sporadic pancreatic cancer. Goggins et al, found BRCA2 mutations in 3/41 (7%) of patients with pancreatic cancer (of which two had the 6174delT mutation) (Goggins et al. 1996), and Ozcelik et al identified 2/42 (4.9%) cases, apparently none with a family history. Ozcelik et al also found the BRCA2 6174delT mutation in 4/39 (10%) patients of Jewish descent with pancreatic cancer and no family history. This prevalence level was significantly higher than the prevalence of the 6174delT mutation in the general Ashkenazi population (1.4-2.5%). The cumulative risk for pancreatic cancer by the age of 75 years in BRCA2 mutation carriers was estimated to be 7% compared to 0.85% for the general population (Ozcelik et al. 1997).

In a study Lal et al investigated germline mutations (CDKN2A, BRCA1, BRCA2, hMSH2, and hMLH1) in 102 consecutive cases of pancreatic cancer and ascertained levels of risk based on the family history. Thirty-eight (37%) patients were characterized as high or intermediate risk, five (13%) of whom had a germ-line mutation -one in CDKN2A (I49S); one in BRCA1 (5382 insC); and three in BRCA2 (6174delT). All four BRCA1 and BRCA2 mutations were identified in Ashkenazi Jewish patients, of whom

there were 14 (14%) in the study. Four of the five mutation carriers had a strong family history associated with family cancer syndromes linked to the relevant mutated genes. Mutations were not identified in patients in whom a family history of pancreatic cancer was the solitary risk factor and a mutation was found in only one patient with early-age disease onset. The absence of germ-line mutations in most high- and intermediate-risk cases indicates that there are other unidentified genes predisposing to pancreatic cancer (Lal et al. 2000).

In a collaborative study by Hahn et al, 26 European familial pancreatic cancer families (64 patients) were analysed for BRACA2 mutations. Three families were found to carry a frameshift mutation in the BRACA2 gene. The group also identified two families with sequence variants of the gene. In conclusion of the study, Hahn et al recommended BRACA2 genetic testing and counselling strategies in families with familial pancreatic cancer as their data supported an important role for BRACA2 mutations in this subgroup of patients (Hahn et al. 2003).

Ataxia Telangiectasia

AT is an autosomal recessive syndrome with an estimated frequency of one per 40,000 to one per 300,000 births. AT is characterized by a progressive cerebellar ataxia, oculo-cutaneous telangiectasias, oculo-motor apraxia, respiratory tract infections and an increased risk of lymphoproliferative malignancies in particular. There is increased radiosensitivity and an overall risk of cancer of around 50- to 150-fold in homozygotes and perhaps a 3-fold risk in heterozygotes (Swift et al. 1987; Swift et al. 1991).

The gene responsible is the ATM gene located on chromosome 11q22-23 and between 0.2% and 1% of the general population are heterozygous carriers of an altered ATM allele (Savitsky et al. 1995). Irradiation-induced double strand DNA damage results in ATM binding to and serine 15 phosphorylation of p53, thereby contributing to the activation and stabilization of p53 (Khanna et al. 1998) and ATM is also directly responsible for the phosphorylation of BRCA1 in response to ionizing radiation (Cortez et al. 1999).

Seven cases of pancreatic cancer were reported in blood relatives of 110 white families with AT compared to 3.3 expected cases and there was one case amongst the spouse controls (1.3 expected) but these differences were not statistically significant (Swift et al. 1987). Two recent studies from the Nordic countries and Britain have failed to reveal an increased incidence of pancreatic cancer in AT families (Inskip et al. 1999; Olsen et al. 2001).

Hereditary Non-Polyposis Colon Cancer

HNPCC is the most common genetic predisposition to colorectal cancer. It is an autosomal dominant syndrome characterized by early-age onset of right-sided colon cancers and in 25% of cases is associated with an increased incidence of multiple cancers (Lynch et al. 1985). Lynch syndrome II is characterized by site-specific colorectal cancer with early onset but also includes an excess of extra-colonic cancers including gynaecological (endometrial, ovarian and breast) cancers and transitional urothelial tumours as well as gastric, small bowel and pancreatic cancers (Lynch et al.

1997). The disease is caused by mutations in one of several DNA mismatch-repair genes (notably hMLH1, hMSH2 and hMSH6 but also hPMS1 and hPMS2) (Akiyama et al. 1997; Peltomaki et al. 1997). An ATM polymorphism (1853D) may modulate the penetrance of hMSH2 and hMLH1 (Maillet et al. 2000). The exact risk of pancreatic cancer in the Lynch II syndrome is poorly documented.

Six cases with pancreatic cancer were identified amongst 293 putative gene carriers from 40 Finnish HNPCC kindreds. The cumulative risk (to the age of 80 years) for pancreatic and biliary tract cancers was estimated to be 17.5% (Aarnio et al. 1995). Lynch et al reported one HNPCC family with carcinoma of the bile duct, urothelial tumours and an extremely early-onset carcinoma of the pancreas, in patients considered to have inherited the deleterious genotype (Lynch et al. 1991).

Familial Adenomatous Polyposis

FAP is an autosomal dominant disease with near complete penetrance. It is characterized by the appearance of >100 (usually 1000s of) colorectal polyps from an early age that invariably undergo malignant transformation and lead to death from metastases if not removed by surgical resection. Extra-colonic manifestations of FAP include epidermoid cysts, congenital hypertrophy of the retinal pigment epithelium, multiple gastric and duodenal polyps and desmoid tumours. The cause of FAP (in at least 80% of families) is a germline functional mutation of the tumour-suppressor APC gene located on 5q21. Mutations are mostly due to point mutations, usually leading to truncation and also

frameshift mutations (Groden et al. 1991; Kinzler et al. 1991) or genomic rearrangements (Su et al. 2000).

The APC protein regulates of β-catenin regulated transcription. β-catenin can associate with Tcf/Lef DNA-binding proteins and can form DNA sequence specific transcription complexes. The APC protein associates with β-catenin together with glycogen synthase kinase-3 (GSK3) and axin or conductin thereby leading to its degradation. An attenuated form of FAP, in which patients develop fewer tumors and at an older age, is associated with an allele of APC carrying a mutation on exon 9 (APC_{AS9}) (Su et al. 2000).

There appears to be a relatively small increased risk of pancreatic cancer in FAP, although the exact risk is not known due to rather few studies. Data from the Johns Hopkins registry revealed an increased RR of 4.46 (95% CI, 1.2-11.4%) for pancreatic adenocarcinoma (Giardiello et al. 1993).

Li-Fraumeni

The Li-Fraumeni syndrome is an autosomal dominant condition with a predisposition to the early onset of sarcomas, breast cancer, brain tumors, lung cancer, adreno-cortical tumours and leukaemias (Strong et al. 1987). The causative gene in many cases is the TP53 tumour-suppressor gene that encodes for the p53 protein and lies on chromosome 17p13. p53 has an absolutely critical role in the control of the cell cycle and is centrally involved in DNA repair processes and apoptosis (Malkin et al. 1990; Srivastava et al. 1990).

Many Li-Fraumeni patients have mutations in exons 5 to 8 of TP53 but around 50% patients have no germline mutation in this gene (Frebourg et al. 1995). The presence of a germline missense mutation in the core DNA binding domain produces a much more highly penetrant phenotype with a higher incidence of cancers and diagnosis at an earlier age (Birch et al. 1998).

The extent of pancreatic cancer involvement in the Li-Fraumeni syndrome is unclear due to limited data. In a study of 24 kindreds from the USA in 1988, one case of pancreatic cancer was seen in each of three families and two cases were seen in a fourth family (Li et al. 1988). A follow-up study was reported in 1998 of these 24 kindreds diagnosed between 1968 and 1986. Two- hundred family members developed a primary tumour, seven of which were pancreatic cancers. Thirty (15%) of these patients subsequently developed another cancer including a single case of pancreatic cancer (Hisada et al. 1998).

Diabetes Mellitus and Obesity

There is much controversy surrounding the association between diabetes and pancreatic cancer. Many studies have been carried out to try to unravel the relationship between pancreatic cancer and diabetes.

There is an incompletely understood relationship between pancreatic cancer and diabetes mellitus. In 1995 Evans et al reported a large pedigree with nine cases of PDAC and an autosomal dominant pattern of inheritance and in whom diabetes mellitus and pancreatic exocrine insufficiency developed prior to the onset of pancreatic cancer (Evans et al. 1995). Members of the family developed pancreatic cancer at an earlier and earlier age in subsequent generations (anticipation). Five of the nine cases with cancer had diabetes mellitus >10 years prior to the development of pancreatic cancer and both conditions were diagnosed concomitantly in the remainder. A meta-analysis in 1995 by Everhart et al of 20 case-control and cohort studies of diabetics revealed a relative risk of 2.1 (95% CI, 1.68-2.8) for pancreatic cancer. Diabetes mellitus of >5 years standing carried the same relative risk as those diagnosed sooner. The increased risk was restricted to patients with non-insulin dependent diabetes mellitus. A study by Kath et al of 2,720 insulin- dependent diabetic patients found four cases of pancreatic cancer, which was not significantly different from the incidence in non-diabetics (Kath et al. 2000).

This significant association between diabetes mellitus and pancreatic cancer is supported by more studies. In 1997, Wideroff et al investigated 109,581 individuals hospitalized

with diabetes mellitus in Denmark. The standardized incidence ratio (SIR) for pancreatic cancer was 2.1 (95% CI, 1.9-2.4) with a follow-up time of 1-4 years; the SIR declined to 1.3 (95% CI, 1.1-1.6) after 5-9 years of follow-up. Similar findings were reported from Sweden (Chow et al. 1995). Calle et al in 1998 examined the association of pancreatic cancer mortality (2,953 deaths) and diabetes mellitus (mostly non-insulin-dependent) of at least one year's duration in a cohort of 1,089,586 subjects (Calle 1998). Diabetes was significantly related to pancreatic cancer mortality in both men (relative risk = 1.49; 95% CI, 1.25-1.77) and women (1.51; 95% CI, 1.24-1.85). In 1999, Silverman et al found that patients who had diabetes mellitus for $\geq$10 years had a 50% increased risk, while patients with diabetes of one-year duration had a 30% increased risk of pancreatic cancer (Silverman et al. 1999). Interestingly, Gapstur et al reported a 2.2-fold increased risk of pancreatic cancer in diabetic patients with high post-load plasma glucose level compared to those with lower levels (Gapstur et al. 2000).

There are also studies disputing the increased risks of pancreatic cancer by diabetes mellitus. Some authors have indicated that diabetes mellitus seem not to be a risk factor (Gullo 1999).

Rulyak et al conducted a nested case-control study including 251 members of 28 families with familial pancreatic cancer (families with at least 2 first- or second –degree members with pancreatic cancer) looking at amongst other risk factor, diabetes and whether this increases the risk of pancreatic cancer. This group concluded that diabetes

was not associated with increased pancreatic cancer risk in familial pancreatic cancer families (Rulyak et al. 2003).

Conversely, Bonelli et al reported the risk of pancreatic cancer associated with diabetes mellitus to be 2.89. When the data was analysed further based on the diabetic treatment administered, they found the overall risk was higher for subjects treated with insulin (relative risk= 6.49) than those treated with oral hypoglycaemic drugs (relative risk= 2.12). Furthermore, after 5 years from the diagnosis of diabetes, the increased risk for pancreatic cancer associated to diabetes persisted only for patients treated with insulin- a 7-fold increased risk for developing pancreatic cancer as compared with those treated with oral antidiabetics (Bonelli et al. 2003).

A large study looking at fasting serum glucose levels in a Korean cohort of over a million individuals with a 10 year follow up period showed a clear association between hyperglycaemia and pancreatic carcinoma (Jee et al. 2005). It still remains unclear whether diabetes is a risk factor for pancreatic cancer or an early symptom, a recent paper by Chari et al (Chari et al. 2008) suggested that only recent onset diabetes increases risk. However, this remains controvertial as the incidence of diabetes at earlier time points (before diagnosis with cancer) was still very high in the cohort studies.

The link between pancreatic cancer and obesity has also been studied. Pan et al in Canada conducted a large (21022 incident cases of 19 types of cancers and 5039 controls) population-based, case-control study and reported a positive association

between obesity and pancreatic cancer. The overall population attributed risk for pancreatic cancer related to weight for overweight versus obese patients was -0.33% and 7.11% respectively (Pan et al. 2004).

Obesity (defined as body mass index of at least 30 kg/m^2) has been positively linked with pancreatic cancer. A Swedish study observed, compared to normal weight men, there was an elevated risk of pancreatic cancer in non-smoking obese men compared to no overall increased risk when the group was not stratified by smoking status (Samanic et al. 2006).

The findings are in consensus with the earlier reports from Michaud et al (suggesting ana 50-70% increased risk of pancreatic cancer in obese patients) (Michaud et al. 2001) and Isaksson et al that adult weight gain was associated with a 50% increased risk of PDAC (Isaksson et al. 2002).

However, a meta-analysis carried out by Berrington et al in 2003 involving 6391 cases of pancreatic cancer reported a weak link between obesity and pancreatic cancer- a relative risk per unit increase in body mass index (5 kg/m^2) of 1.16 (Berrington de Gonzalez et al. 2003). This weak link was further supported by a study examining the relationship between anthropometry, physical activity and risk of pancreatic cancer in published in 2006. The relative risk per unit increase in body mass index (5 kg/m^2) was 1.09 (Berrington de Gonzalez et al. 2006).

Origin of Pancreatic Ductal Adenocarcinoma

The pancreas has both endocrine and exocrine functions. The exocrine pancreas makes up to over 95% (acinar cells 85%, ductal cells 10%) of the total volume of the gland and the endocrine cells occupy only 1-2% of the pancreas. PDAC is the most common type of cancer of the pancreas accounting for greater than 85% of pancreatic neoplasms.

PDAC cells morphologically resemble ductal epithelial cells but there is much debate as to the origin and nature of these tumours. The morphological appearance of PDAC suggests that the cells of origin are of ductal epithelial cell lineage. However, there has also been some evidence of islet cells transformation into PDAC (Pour et al. 1999).

Pancreatic Intraepithelial Neoplasia

Pancreatic Intraepithelial Neoplasia (PanIN) is the terminology adopted by the Pancreas Think Tank in 1999 to reflect the potential progression to neoplasia of various precursor pancreatic lesions. These lesions have been observed in pancreas specimens with ductal carcinomas, chronic pancreatitis and even in normal pancreases. PanIN is subclassified into PanIN-1A, PanINB-1B, PanIN-2 and PanIN-3 based on the degree of cytological and architectural atypia present. In summary, PanIN-1a and Pan-1B are those lesions with slight or no atypia; PanIN-2 designates lesions with moderate atypia; and PanIn-3 designates those lesions with severe atypia. Table 3 explains the cytological and architectural criteria in more detail.

Normal	The normal ductal and ductular epithelium is a cuboidal to low-columnar epithelium with amphophilic cytoplasm. Mucinous cytoplasm, nuclear crowding and atypia are not seen.
Squamous (transitional) metaplasia	A process in which the normal cuboidal ductal epithelium is replaced by mature squamous or transitional epithelium without atypia.
PanIN-1A	**(Pancreatic Intraepithelial Neoplasia 1-A)**: These are flat epithelial lesions composed of tall columnar cells with basally located nuclei and abundant supranuclear mucin. The nuclei are small and round to oval in shape. When oval the nuclei are oriented perpendicular to the basement membrane. It is recognized that there is considerable histologic overlap between non-neoplastic flat hyperplastic lesions and flat neoplastic lesions without atypia.
PanIN-1B	**(Pancreatic Intraepithelial Neoplasia 1-B)**: These epithelial lesions have a papillary, micropapillary or basally pseudostratified architecture, but are otherwise identical to PanIN-1A.
PanIN-2	**(Pancreatic Intraepithelial Neoplasia 2):** Architecturally these mucinous epithelial lesions may be flat or papillary. Cytologically, by definition, these lesions must have some nuclear abnormalities. These abnormalities may include some loss of polarity, nuclear crowding, enlarged nuclei, pseudo-stratification and hyperchromatism. These nuclear abnormalities fall short of those seen in PanIN-3. Mitoses are rare, but when present are non-luminal (not apical) and not atypical. True cribriforming luminal necrosis and marked cytologic abnormalities are generally not seen, and when present should suggest the diagnosis of PanIN-3.
PanIN-3	**(Pancreatic Intraepithelial Neoplasia 3):** These lesions are usually papillary or micropapillary -they may rarely be flat. True cribriforming, budding off of small clusters of epithelial cells into the lumen and luminal necroses all suggest the diagnosis of PanIN-3. Cytologically, these lesions are characterized by a loss of nuclear polarity, dystrophic goblet cells (goblet cells with nuclei oriented towards the lumen and mucinous cytoplasm oriented toward the basement membrane), mitoses which may occasionally be abnormal, nuclear irregularities and prominent (macro) nucleoli

Table 3: Existing Pancreatic Intraepithelial Neoplasia nomenclature (Hruban et al. 2007)

Linking PanIN and Cancer

A number of pieces of evidence have been put forward in support of the hypothesis PanIN is the precursor lesion for pancreatic cancer. A number of studies has proven that histological examination of pancreases with PDAC and normal pancreases reveal ductal papillary hyperplasia are more common in the former group of pancreases (Cubilla et al. 1976; Kozuka et al. 1979; Kloppel et al. 1980; Chen et al. 1985; Luttges et al. 1999). The incidence of ductal changes in the pancreas increases with age. Lastly, hyperplastic ductal lesions may harbour mutations of the K-ras gene which is are characteristic of ductal adenocarcinomas (Yanagisawa et al. 1993; Tada et al. 1996; Moskaluk et al. 1997; Matsubayashi et al. 1998; Terhune et al. 1998; Heinmoller et al. 2000).

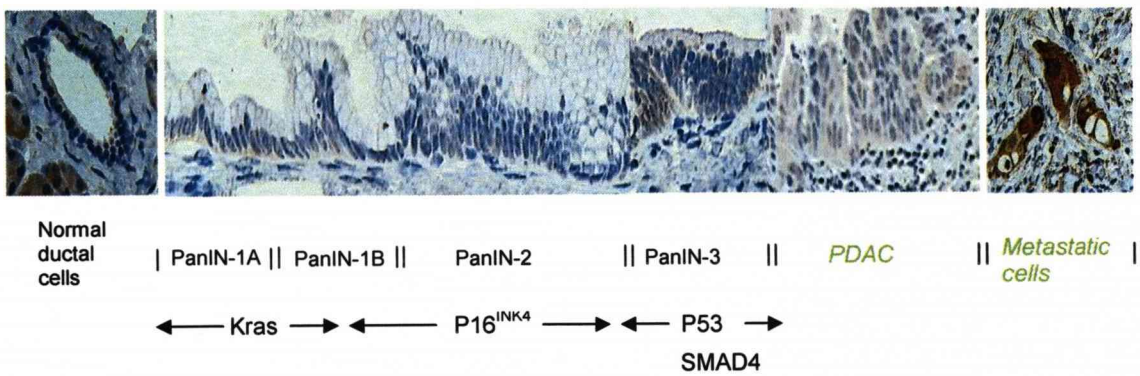


Figure 6: Demographic progression model for pancreatic cancer

(Illustrations by A Shekouh, taken with permission)

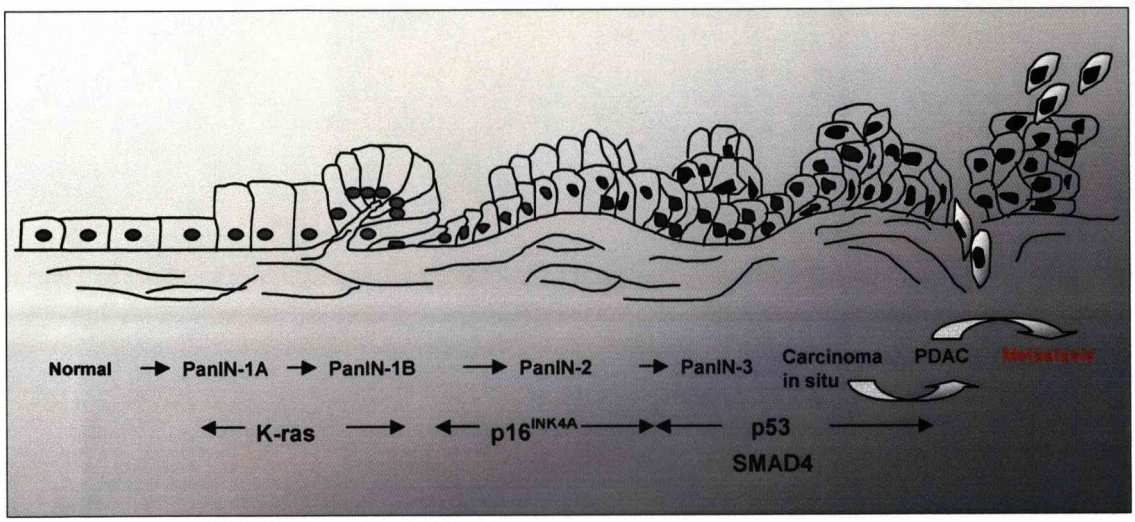


Figure 7: Progression model for pancreatic cancer.

(Illustrations by A Shekouh, taken with permission)

Normal duct epithelium progresses to infiltrating cancer (left to right) through a series of histologically defined precursors (PanINs). The point mutations in the K-ras gene occur early, inactivation of the p16 gene at an intermediate stage, and the inactivation of p53, SMAD4 occur relatively late.

Grading and Staging of Pancreatic Ductal Adenocarcinoma

Grading Pancreatic Ductal Adenocarcinoma

The grading of PDAC, as with any other cancers, is a microscopic issue. The pathologist with a surgically resected or biopsy specimen, will be able to grade the tumour according to the microscopic appearances of the cells, taking into account glandular differentiation, mucin production, nuclear atypia and mitotic activity (Kloppel et al. 1985).

The grading category falls into three divisions- well, moderately and poorly differentiated and the aggressiveness of the tumour usually follows accordingly. Unfortunately, the process of grading a specimen for PDAC is subjective, not reproducible, and very dependent on the experience of the observer. The grading system used in most Western countries is the WHO organization Table 4 (Kloppel et al. 1996).

Table 4: Histological grading of Pancreatic Ductal Adenocarcinoma (Kloppel et al. 1996)

Differentiation	Duct structures	Nuclei	Mitotic figures per 10 high power fields*	Mucin production
Well	Well formed	Basal	< 5	Marked
Moderate	Some well formed	Loss of polarization, anisonucleosis	5- 10	Variable
Poor	Very irregular or absent	Marked anisonucleosis, clumped chromatin	>10	Minimal

Grading of pancreatic cancer has been linked to survival benefits following surgical resection. Meyer et al confirms the survival benefit in a larger number of patients (91 patients who had undergone pancreatic resections), with reports of a 5-year survival rate of 32% (13/91 patients) for well-differentiated tumours compared with 0% (14/91 patients) for poorly differentiated tumours (Meyer et al. 2000).

Lüttges et al reported that tumour grade is a highly significant prognostic factor- especially when based on the observation field with the lowest degree of differentiation. The mean survivals were 27.6 months, 18.2 months and 8.1 months for the tumour grades well, moderately and poorly differentiated respectively (Luttges et al. 2000).

From Italy, Benassai reported much improved survival of patients with resected well differentiated pancreatic ductal adenocarcinoma (4 patients) compared with patients with moderately (21 patients) and poorly differentiated (50 patients) tumours- (median survival 54, 33, 16 months respectively; 5-year survival 75%, 42.9%, 4% respectively) (Benassai et al. 2000). The actuarial 5-year survival rate for this group of patients following resection of their tumour was 18.7%. The survival rates were also influenced by the lymph node status, DNA content of the pancreatic cancer cells, the pathological margins and the tumour diameter.

Other authors have reported actuarial survival rates in patients with resected pancreatic ductal adenocarcinoma varying from 6.8-24% (Cameron et al. 1993; Baumel et al. 1994; Bramhall et al. 1995; Nitecki et al. 1995; Wade et al. 1995; Benassai et al. 2000). Again, the patients with smaller tumours, negative lymph nodes, diploid tumours, negative resection margins and well-differentiated tumours achieved better survival rates than their counterparts.

Grade, stage and nodal status are interrelated making survival analysis a complex issue to discuss. This is an important and interesting area, but for the purpose of this thesis the most important observation is that the earlier tumours are detected the better the chances of survival.

Staging of Pancreatic Ductal Adenocarcinoma

The diagnosis of PDAC is dependent upon physical examination, imaging, and/or surgical exploration. Ideally, there should be histological or cytological confirmation of the disease, although this may not be attainable in all the cases.

The most widely used staging classification for PDAC is published by the Union Internationale Contre le Cancer (UICC). The current classification takes into account the following factors- primary tumour (T), lymph node metastasis (N) and distant metastasis (M) Table 5 and Table 6. There is a different staging classificatification in Japan which will not be discussed in any further detail. Appropriate staging in the diagnosis of pancreatic ductal adenocarcinoma is very important to avoid unnecessary exploratory surgery, which is time consuming, expensive and inflicts physical and psychological trauma to the patient.

TX	Primary tumour cannot be assessed	NX	Regional lymph node cannot be assessed	MX	Distant metastasis cannot be assessed
T0	No evidence of primary tumour	N0	No regional lymph node metastasis	M0	No distant metastasis
Tis	Carcinoma in situ	N1	Regional lymph node metastasis	M1	Distant metastasis
T1	Tumour limited to the pancreas, ≤ 2cm in greatest diameter				
T2	Tumour limited to the pancreas, ≥ 2cm in greatest diameter				
T3	Tumour extends beyond pancreas, but without involovement of coeliac axis or superior mesenteric artery				
T4	Tumour involves coeliac axis or superior mesenteric artery				T= tumour N= Nodes M= Metastasis

Table 5: UICC TNM Clinical Classification of Pancreatic Cancer 2006 (Sobin et al. 2006)

Stage	T category	N category	M category
0	Tis	N0	MO
IA	T1	N0	M0
IB	T2	N0	M0
IIA	T3	N0	M0
IIB	T1, 2, 3	N1	M0
III	T4	Any N	M0
IV	Any T	Any N	M1

T=Tumour, N= Lymph nodes, M= Metastasis

Table 6: UICC Stage Classification for Pancreatic Ductal Adenocarcinoma (Sobin et al. 2006)

Current clinical practice in the staging of pancreatic ductal adenocarcinoma includes the use of biochemical analysis of blood samples for tumours markers carcinoembryonic antigen (CEA) and CA19-9. These tumour markers may confirm the suspicion of a pancreatic malignancy but they may also be falsely elevated in the presence of benign conditions of the pancreas and liver.

Most pancreatic lesions are initially noticed on USS and/or ERCP. These initial investigations may be followed by more detailed imaging with computed tomography and/or magnetic resonance scans.

Prior to undertaking potentially curative surgery, there are pancreatic units performing laparoscopy to rule out the possibility of metastatic spread of the cancer. In potentially resectable pancreatic cancer determined by conventional imaging studies, surgeons have found the spread of the cancer outside the pancreas in 10-34% of patients (Conlon et al. 1996; Friess et al. 1998; Jimenez et al. 2000).

Conventional Diagnostic Modalities of Pancreatic Ductal Adenocarcinoma

Rapid advances in radiological imaging have enabled clinicians to greatly reduce the number of patients undergoing unnecessary surgery in an attempt to cure them of their pancreatic malignancy. With the aid of current diagnostic aids, the number of patients undergoing pancreatic surgery with the intention of cure has been reduced to less than 10% (Allema et al. 1995; Nitecki et al. 1995).

Despite major advances in laboratory diagnosis and radiological technology, the sensitivity and specificity of these tests are still not high enough for them to be used as reliable screening tools. The variation in sensitivity and specificity of the imaging and laboratory modalities used are shown in the tables to follow.

Tumour Markers

CA 19-9 is one of the most commonly used tumour markers for PDAC but there is a variation in the sensitivity. Steinberg et al compared the sensitivity and specificity of CA19-9 and CEA results for 37 patients with biopsy proven pancreatic adenocarcinoma- 14 patients with resectable disease and 23 patients with unresectable disease. 157 controls were also analysed- 48 patients with benign pancreatic disease, 34 patients with non-pancreatic sources of abdominal pain, 58 patients with benign jaundice, 7 patients with nonpancreatic malabsorption, and 10 patients with renal failure on dialysis. The group reported the sensitivity for CA19-9 (>75U/ml) was 86.5% compared to CEA

(>5ng/ml) which was 48.4%. The specificity of CA19-9 was 92.5% versus 87.3% for CEA (Steinberg et al. 1986).

A collective study from 7 institutions in Japan by Furukawa et al analysing small pancreatic adenocarcinoma (2cm or less in greatest dimension) reported the sensitivity for CA19-9 to be 58.1% (18 of 31 patients). This compared unfavourable to USS, EUS, CT and ERCP which had sensitivity in this study reported as 64.5%, 73.7%, 64.5% and 93.1% respectively (Furukawa et al. 1996).

Serum CA 19-9 level may also be raised in other malignancies such as adenocarcinoma of the stomach, colon and the hepatobiliary system as well as in obstructive jaundice, acute cholangitis and worst of all in chronic pancreatitis (Tanaka et al. 2000). Other possible tumour markers such as CEA, SPAN-1, CA 50 and TAG-72, have even lower diagnostic efficiencies compared to CA 19-9 (Kuno et al. 1994; Pasquali et al. 1994; Safi et al. 1996) (Table 9). For small resectable tumours both the sensitivities and specificities of all the tumour markers are even worse (Neoptolemos et al. 1996).

Table 7: The accuracy of tumour markers in diagnosing pancreatic ductal adenocarcinoma

Series	Sample	No. in series	CA19-9	CEA	Others
Uno et al (Uno et al. 2000)	PJ	40- 15 PC - 25 CP	Sen 5/10 (33) Spec 24/25 (92)	Sen 2/15 (13) Spec 24/25 (96)	
Tanaka et al (Tanaka et al. 2000)	Serum	32- 11 PC - 21 CP	Sen 7/11 (64) Spec 20/21 (95)		
Ventrucci et al (Ventrucci et al. 1998)	Serum	127- 46 PC - 53 CP - 28 AP	Sen 29/46 (63) at Spec (95)		CA-50: Sen 29/46 (63) at Spec (95) CA 242: Sen 25/46 (54) at Spec (95)
Safi et al (Safi et al. 1996)	Serum	647- 347 PC - 300 CP - 786 Benign disease	Sen 296/347(85) Spec 951/1086 (87)		
Kuno et al (Kuno et al. 1994)	Serum	367- 47 PC - 310 Controls*	Sen 41/47 (87) Spec 254/310 (82)	Sen 26/47 (55) Spec 279/310 (90)	SPAN 1: Sen 42/47 (89) Spec 260/310 (84)
Pasquali et al (Pasquali et al. 1994)	Serum	105- 58 PC - 45 CP	Sen 47/58 (81) Spec 43/45 (95)		TAG-72: Sen 26/58 (45) Spec 43/45 (95)
Steinberg et al (Steinberg et al. 1986)	Serum	194- 37 PC - 157 Controls**	Sen 32/37 (87) Spec 136/147 (93)	Sen 15/31 (48) Spec 96/110 (87)	

PC = pancreatic cancer; CP = chronic pancreatitis; AP = acute pancreatitis; PJ = pancreatic juice; Sen = sensitivity (the rate of pick-up of the disease in a test); Spec = specificity (the rate at wich a test can eclude the possibility of a disease). Numbers in parentheses are percents. *310 Controls- 70 chronic pancreatitis, 16 other pancreatic disease, 60 hepatobiliary disease, 164 other benign disease. **157 Controls- 48 benign pancreatic disease, 34 nonpancreatic sources of abdominal pain, 58 benign jaundice, 7 nonpancreatic malabsorption, 10 renal failure on dailysis

Imaging Modalities

Rosch et al recently compared in a prospective manner the accuracy of USS, computed CT, ERCP and EUS in 184 in-patients who were referred for evaluation of suspected pancreatic disease. The final diagnoses were normal pancreas in 36, chronic pancreatitis without a focal inflammatory mass in 53 or with a focal inflammatory mass in 18 and pancreatic malignancy in 77 cases. The sensitivity for pancreatic disease for TUS was 94% but the specificity was only 35%. The sensitivity and specificity was 91% and 78% for CT, 89% and 92% for ERCP and 93% and 94% for EUS, respectively. There was no difference in the sensitivity between the imaging tests in cancer and chronic pancreatitis (Rosch et al. 2000). Adamek et al prospectively compared magnetic resonance cholangiopancreatography (MRCP) with ERCP in 124 patients with a strong clinical suspicion of pancreatic cancer: 37 (30%) had pancreatic carcinoma, 17 (14%) had other neoplastic pancreatic diseases, 57 (46%) had chronic pancreatitis and in 13 (10%) the main pancreatic duct was normal. The sensitivity of MRCP for diagnosing pancreatic cancer was 84% and its specificity was 97%, compared to 70% and 94%, respectively for ERCP. The diagnosis of malignancy was confirmed by comparing the MRCP/ERCP diagnosis with histopathological evidence using laparotomy or ultrasound guided biopsy. In patients lacking histological evidence, a follow up of at least 12 months was used as reference (Adamek et al. 2000). In an important prospective study of ERCP in 14 asymptomatic patients with a history of FPC, Brentnall et al found suspicious lesions in seven cases, all of whom were shown to have pancreatic ductal dysplasia (pancreatic intraepithelial neoplasia – PanIN - grades 2 and 3) by histology of the surgically resected specimens (Brentnall et al. 1999).

The diagnosis of pancreatic cancer using endoscopic brush cytology of the pancreatic duct has a sensitivity of 67-76% and a specificity of 83-100% (Vandervoort et al. 1999) and in the case of biliary duct brushings the sensitivity is 36-50% and the specificity is 82-97% (Glasbrenner et al. 1999; Vandervoort et al. 1999) (Table 8).

Müller et al reported a much higher sensitivity (93%) using EUS for tumour detection than either dynamic CT (53%) or magnetic resonance imaging (MRI; 67%) (Muller et al. 1994). The difference was apparently more striking for tumours <20mm with sensitivities of 90%, 40% and 33% for EUS, dynamic CT and MRI respectively. The overall accuracy for the diagnosis of malignant lesions was 86% (EUS), 67% (CT), and 84% (MRI). The final diagnosis for this group of patients were 16 PDAC, 6 oeriampullary tumour, 2 benign neoplasms, 9 inflammatory tumours, and no tumour. The no tumour group of patients were followed up for a period of 9-24 months (Muller et al. 1994). Midwinter et al reported that EUS demonstrated 23/24 (96%) pancreatic cancers compared with only 19/24 (78%) using spiral CT (Midwinter et al. 1999) Table 9.

Voss et al evaluated EUS-fine needle aspiration biopsy (EUS-FNAB) in 73 cases (51 PDAC, 8 neuroendocrine tumours, 6 various neoplasms and 8 pancreatitis). The sensitivity, specificity, positive and negative predictive values for differentiating pancreatic adenocarcinoma from chronic pancreatitis was 81%, 88 %, 98% and 39% respectively (Voss et al. 2000).

Positron emission tomography (PET) with radiolabelled flurodeoxyglucose has a sensitivity of 71-87% and a specificity of 50-64% for detecting malignant pancreatic lesions (Mertz et al. 2000; Sendler et al. 2000). Hosten et al have produced a preliminary report to suggest that sensitivity for smaller pancreatic lesions may be increased by using image fusion of PET with CT (Hosten et al. 2000).

Table 8: The accuracy of brush cytology and fine needle aspiration biopsy in the diagnosis of Pancreatic Ductal Adenocarcinoma

Series	No. in series	EUS-FNAB	Brush cytology (Bile Duct)	Brush cytology (Pancreatic Duct)
Voss et al (Voss et al. 2000)	69 (59 PC, 10 CP)	Sen 48/59 (81) Spec 7/8 (78)		
Glasbrenner et al (Glasbrenner et al. 1999)	52 (31 PC, 21 Benign)		Sen 11/31 (36) Spec 19/21 (91)	
Vandervoort et al (Vandervoort et al. 1999)	90 (67 PC, 23 CP)		Sen 23/46 (50) Spec 9/11 (82)	Sen 14/21 (67) Spec 12/12 (100)
Strum et al (Sturm et al. 1999)	112 (38 PC, 74 Benign)		Sen 8/38 (21) Spec 72/74 (97)	
Van Laethem et al (Van Laethem et al. 1995)	40 (24 PC, 16 CP)			Sen 13/17 (76) Spec 10/12 (83)

PC = pancreatic cancer; CP = chronic pancreatitis; Sen = sensitivity; Spec = specificity; EUS-FNAB = endoscopic ultrasound fine needle aspiration biopsy. Numbers in parentheses are percents.

Table 9: Comparison of abdominal ultrasound, endoscopic ultrasound, endoscopic retrograde cholangiopancreatography, computed tomography, magnetic resonance imaging and positron emission tomography for the diagnosis of pancreatic cancer

Series	No. in series	US	EUS	ERCP/MRCP	CT	MRI	PET
Mertz et al (Mertz et al. 2000)	31		Sen 29/31 (93) Spec 3/4 (75)		Helical: Sen 16/31 (53), Spec 1/4 (25)		Sen 27/31 (87) Spec 2/2 (50)
Sendler et al (Sendler et al. 2000)	42	Sen 18/32(56) Spec 5/10 (50)			Helical: Sen 23/31(74) Spec 8/14 (57)		Sen 22/31 (71) Spec 7/11 (64)
Rösch et al (Rosch et al. 2000)	130 (77PC, 53 CP)		Sen 66/77 (86) Spec 46/53 (87)	ERCP: Sen 62/77 (81) Spec 45/53 (85)	Sen 62/77 (81) Spec 44/53 (83)		
Adamek et al (Adamek et al. 2000)	94 (37 PC, 57 CP)			MRCP: Sen 31/37 (84) Spec 50/57 (88) ERCP: Sen 26/37 (70) Spec 51/57 (89)			
Phoa et al (Phoa et al. 1999)	56 PC				Spiral: Sen 49/51(91)		
Midwinter et al (Midwinter et al. 1999)	26		Sen 25/26 (96)		Spiral Spiral: Sen 21/26 (81)		
Müller et al (Muller et al. 1994)	49		Sen 31/33 (94) Spec 16/16 (100)		Sen 22/32 (69) Spec 9/14 (64)	Sen 19/23 (83) Spec 2/2 (100)	

US= abdominal ultrasound; EUS= endoscopic ultrasound; CT = computed tomography; MRI = magnetic resonance imaging; PET = Positron Emission Tomography; PC = pancreatic cancer; CP = chronic pancreatitis; Sen = sensitivity; Spec = specificity. Numbers in parentheses are percent

Molecular Diagnosis of Early Pancreatic Pancreatic Ductal Adenocarcinoma in High Risk Patients

Screening Of Pancreatic Cancer Requirements

The key determinants for screening are (i) the importance of the disease; (ii) the prevalence of the disease; (iii) the accuracy of the screening methodology; and (iv) the cost-benefit ratio of the screening. Whilst there is no doubting the importance of pancreatic cancer its prevalence is not high enough per se to justify the use of screening (Neoptolemos et al. 1996).

The fundamental requirement for screening is that the benefits for the true positive population must outweigh the harm done to the false positive population. In the case of pancreatic cancer true positives may (or possibly may not) be saved from a horrible death (by removing the tumour surgically), but false positives will undergo an unnecessary and possibly life threatening surgical procedure. In very general terms we can therefore say that for screening to be ethical, the true positive: false positive (TP: FP) ratio must be ≥ 1.0, since the proportion of false positives must not exceed that of true positives by any large amount. If the TP: FP ratio is $<<1.0$ then any benefit derived from screening is lost due to the excess morbidity (and mortality), as well as the cost associated with treating a large number of false positives. Assuming, the prevalence of the at-risk cohort to be 10 per 10^5 of the population and the sensitivity and specificity for the conventional screening modalities for pancreatic cancer to be 85%, then the TP: FP ratio is only 6×10^{-4}. Thus with these assumptions any screening programme would be

undermined by the overwhelming number of false positives (1,764 false positives for each true positive).

Screening might be feasible however by focussing on high-risk sub-groups and thereby, in effect, raising the prevalence within the screened group. For pancreatic cancer the key risk factors are increasing age, tobacco smoking, chronic pancreatitis, an inherited predisposition and diabetes mellitus (Finch et al. 1997; Bramhall et al. 1998). Taking into account age, the prevalence of the disease in older patients is still, of itself, not high enough to justify the application of screening, nor even if we take into account the increased risk group associated with smoking (Neoptolemos et al. 1996).

Again assuming a sensitivity and specificity for the conventional screening modalities for pancreatic cancer of 85%, by limiting screening to those aged >40 years, the prevalence of the at-risk cohort increases from 10 per 10^5 of the population to 20 per 10^5 of the population. Unfortunately the TP: FP ratio reduces from $6x10^{-4}$ to only $1x10^{-3}$. We can calculate that the prevalence of the at-risk group would need to be $\geq$15,000 per 10^5 of the population screened in order to achieve a manageable ratio for a TP: FP ratio of $\geq$1.0. Even the use of smoking as an additional risk factor does little to tilt the balance in favour of screening since the relative risk of pancreatic cancer for smoking is only ~2-fold (Silverman et al. 1994). However, by selecting genetically high risk patient groups, screening may be a feasible undertaking to identify early pancreatic cancer lesions.

Groups of individuals at much higher risk have been identified, including patients with (sporadic) chronic pancreatitis and a variety of inherited autosomal dominant or autosomal recessive disorders (Hruban et al. 1999; Efthimiou et al. 2001) (Table 1). Therefore, it may be worth screening in genetically high risk groups, where the risks of developing pancreatic cancer might be doubled or more.

The purpose of screening is to identify patients who have developed pre-malignant pancreatic neoplasia so that they can be cured. The most common situation is likely to involve patients who have background pancreatic pathology (CF, HP and sporadic chronic pancreatitis). The only way in which cure can be achieved by total pancreatectomy. Certainty as to the high malignant risk is essential as even in modern units there is still a substantial morbidity and not insignificant mortality from major pancreatic surgery in patients with chronic pancreatitis (Evans et al. 1997; Beger et al. 1999; Buchler et al. 2000).

Even given high-risk populations the screening methodology has to have a sensitivity and specificity in the order of 95-100%. Given our particular requirements the relative importance of specificity far exceeds that of sensitivity. For example if we assume a 10-fold risk for pancreatic cancer (such as that for chronic pancreatitis) and a sensitivity of 95% then to achieve a TP: TF ratio ≥ 1.0 requires a specificity of 99.9% (actual TP: TF = 2.1). Reducing the specificity from 99.9% to just 99.5% produces an unacceptable TP: TF ratio of 4×10^{-1}. On the other hand increasing the sensitivity to 99% produces only a marginal increase in the TP: TF to 2.2. The situation improves somewhat if the at-risk

population has a 100-fold risk (such as that for Hereditary Pancreatitis and Peutz-Jeghers Syndrome). In this case, given a sensitivity of 95% then a TP: TF =1.0 is achieved with a specificity of 98%.

Thus it appears on theoretical grounds that at least some at-risk groups for pancreatic cancer might be suitable for screening. The extent to which the risk in these groups is defined and the relevance of existing diagnostic tools required for screening needs to be examined in further detail.

Molecular Modalities for Diagnosis of Pancreatic Cancer

It is apparent that the diagnosis of pancreatic cancer at an early curable stage, especially in the presence of chronic pancreatitis is served poorly by conventional modalities. The requirements for successful screening in pancreatic cancer are for a sensitivity of 95% and a specificity of 98% to 99.9% depending on the prevalence of the pre-selected at-risk cohort- this requirement is not fulfilled by the diagnosis of pancreatic cancer by tumour markers analysis or by conventional radiological tests. How well do molecular tests perform to these requirements?

With advances in the understanding of the molecular biology of pancreatic cancer and chronic pancreatitis, new diagnostic modalities are being developed with a potentially infinitely better sensitivity and specificity. There are four genes in particular that are involved in the molecular pathogenesis of pancreatic cancer and which may provide the basis for a molecular screening programme.

K-Ras Oncogene

The RAS protein act as an on-off switch that regulate signal transduction pathways controlling cell growth, differentiation and survival. K-ras is a proto-oncogene located on chromosome 12p12 that codes for the 21kDa GTP-binding protein (p21) synthesized in cytoplasm and localized on the inner surface of the plasma membrane. The wild-type K-ras gene code for proteins that bind guanine diphosphate and guanine diphosphate and possesses GTPase activity but the activated form of the gene has no GTPase activity or reduced affinity for guanosine diphosphate (GDP) and guanosine triphosphate (GTP)

(Bos 1988). They are involved in the signal transduction of activated tyrosine kinase cell surface receptors to downstream signal cascades. Activation of the gene results from point mutations at codon 12, 13 and 61 leading to the substitution of glycine with other amino acids, causing the oncogenic Ras protein to be in a permanently active GTP-bound state. Mutations of K-ras (nearly all on codon 12) occur in 75-100% of pancreatic cancers (Tada et al. 1991; Urban et al. 1993; Berrozpe et al. 1994; Banerjee et al. 1997; Wilentz et al. 1998; Kawesha et al. 2000) Table 10.

K-ras mutations have also been found in phenotypically normal and hyperplastic ductal lesions (Yanagisawa et al. 1993) and in neoplastic lesions <20mm in size, suggesting a very early role in the molecular pathogenesis of PDAC. Lüttges et al studied 35 resection specimens of PDAC and three normal pancreata by single cell microdissection and PCR analysis for mutant (mt) K-ras. All of the primary PDACs had mt-K-ras as well as 106/364 ductal lesions including adenomatoid hyperplasia, (53%), papillary hyperplasia (36%), mucinous hypertrophy (26%) and squamous metaplasia (14%) and with only two exceptions the mutation pattern of the ductal lesions and that of the corresponding primary tumor were identical. Twenty-one (17%) samples from normal ducts also harboured the mt-K-ras, as did three lesions from non-cancerous specimens (Luttges et al. 1999). Tada et al reported that 30/30 (100%) micro-dissected adenocarcinoma samples had mt-K-ras compared to 17/79 (24%) hyperplastic cell samples and none of 12 normal duct specimens (Tada et al. 1996). The overall rate of mt-K-ras in 700 chronic pancreatitis patients collated from the published literature in 2000, was approximately 13% (Lohr et al. 2000). Dergham et al examined 81 cases with

PDAC, seven of whom had a family history of pancreatic cancer. K-ras mutations were not statistically different in patients with (83%) and without a family history of PDAC (70%) but the incidence of p53 over-expression was significantly lower in patients with a family history of cancer (40% vs 72%) (Dergham et al. 1997).

Mutant K-ras can be detected in the blood, stool, bile and pancreatic juice from shed cells and free deoxyribonucleic acid (DNA) (Table 11). The proportion of cases with mt-K-ras is dependent on many factors including the mode of sample collection and storage, the method of assay and the test population. Van Laethem et al were able to detect mt-K-ras in 20/24 (83%) pancreatic juice samples from patients with PDAC, similar to Berthélemy et al who reported mt-K-ras in 17/22 (77%) of patients with pancreatic tumours (Berthelemy et al. 1995). Iguchi et al found mt-K-ras in 12/19 (63%) (Iguchi et al. 1996) and Kondo et al in 6/9 of patients with pancreatic cancer (Kondo et al. 1994; Iguchi et al. 1996). Kondo et al also found that 1/41 (2%) patients with benign pancreatic disorders also had mt-K-ras. Watanabe et al found K-ras mutations in the duodenal aspirate of 25/38 (66%) patients with pancreatic carcinoma and in 12/38 (32%) patients with chronic pancreatitis but not in 42 patients with benign biliary diseases or in 20 control patients (Watanabe et al. 1999). Wilentz et al, however, found mt-K-ras in 3/10 (30%) of duodenal juice samples from patients with benign conditions compared to 29/42 (69%) patients with periampullary cancers (Wilentz et al. 1998). Uehara et al found mt-K-ras in the pancreatic juice of 8/10 (80%) cases with pancreatic cancer, 2/3 (67%) cases with chronic pancreatitis and 1/3 (33%) cases with normal pancreas (Uehara et al. 1999).

Table 10: K-ras Mutations in PDAC Tissues

Series	No. in series	Methodology	Percent with K-RAS mutations
Urban et al (Urban et al. 1993)	12	RFLP	11/12 (92)
Hruban et al (Hruban et al. 1993)	82	RFLP	68/82(83)
Pellegata et al (Pellegata et al. 1994)	35	Sequencing	25/35 (71)
Iguchi et al (Iguchi et al. 1996)	10	SSCP	9/10 (90)
Tada et al (Tada et al. 1996)	30	Sequencing	30/30 (100)
Dergham et al (Dergham et al. 1997)	76	SSCP	64/76 (84)
Banerjee et al (Banerjee et al. 1997)	15	RFLP	15/15 (100)
Wilentz et al (Wilentz et al. 1998)	34	RFLP	22/34 (65)
Ward et al (Ward et al. 1998)	6	RFLP	4/6 (67)
Wenger et al (Wenger et al. 1999)	36	Selective-hybridization	28/36 (7)
Lüttges et al (Luttges et al. 1999)	35	Denaturing gradient	35/35 (100)
Aoki et al (Aoki et al. 2000)	10	Sequencing	8/10 (80)
Slebos et al (Slebos et al. 2000)	61	MASA	46/61 (75)
Kawesha et al (Kawesha et al. 2000)	97	SSCP	73/97 (75)

PC = pancreatic cancer; CP = chronic pancreatitis; Sen = sensitivity; Spec = specificity; SSCP = single-strand confirmatory polymorphism; RFLP = restriction fragment length polymorphism; LCM = laser capture microscopy. Numbers in parentheses are percents

Table 11: Accuracy of mutant K-ras detection in diagnostic clinical specimens

Series	No. in series	Sample	Methodo-logy	K-RAS
Kondo et al (Kondo et al. 1994)	33 (9 PC, 14 Control, 10 CP)	PJ	SSCP	Sen 6/9 (67) Spec 0/24 (100)
Tada et al (Tada et al. 1993)	7 (6 PC, 1 Control, 2 CP)	PJ	RFLP	Sen 6/6 (100) Spec 3/3 (100)
Berthélemy et al (Berthelemy et al. 1995)	74 (22PC, 24 Controls, 29 CP)	PJ	RFLP	Sen 17/22 (77) Spec 53/53 (100)
Iguchi et al (Iguchi et al. 1996)	60 (19 PC, 41 Control)	DA	SSCP	Sen 12/19 (63) Spec 40/41 (98)
Watanabe et al (Watanabe et al. 1999)	138 (38 PC, 100 Control)	DA	MASA	Sen 25/38 (66) Spec 88/100 (88)
Yamashita et al (Yamashita et al. 1999)	22 (13PC, 9 CP)	PJ	SSCP	Sen 13/13 (100) Spec 6/9 (67)
Yamaguchi et al (Yamaguchi et al. 1999)	42 (26 PC, 16 CP)	PJ	RFLP	Sen 21/25 (84)
			Selective-hybridization	Sen 17/26 (65)
Uehara et al (Uehara et al. 1999)	16 (10 PC, 6 Control)	PJ	SSCP	Sen 8/10 (80) Spec 3/6 (50)
Caldas et al (Caldas et al. 1994)	15 (11 PC, 4 CP)	Stool	Selective-hybridization	Sen 6/11 (55) Spec 3/4 (75)
Wenger et al (Wenger et al. 1999)	41 (36 PC, 5 CP)	Stool	Selective-hybridization	Sen 7/36 (19) Spec 3/5 (60)
Yamada et al (Yamada et al. 1998)	19(15PC, 4CP)	Serum	MASA	Sen 9/15 (60) Spec 4/4 (100)
Mulcahy et al (Mulcahy et al. 1998)	23 (21PC, 2 CP)	Serum	RFLP	Sen 17/21 (81) Spec 2/2 (100)
Castells et al (Castells et al. 1999)	81 (44 PC, 37 CP)	Serum	RFLP	Sen 12/44 (27) Spec 35/37 (95)
Theodor et al (Theodor et al. 1999)	31 (20 PC, 11 Control)	Serum	RFLP	Sen 14/20 70) Spec 0/11 (100)

PC = pancreatic cancer; CP = chronic pancreatitis; Sen = sensitivity; Spec = specificity; SSCP = single-strand confirmatory polymorphism; RFLP = restriction fragment length polymorphism; MASA= mutant specific allele amplification; PJ = pancreatic juice, DA = duodenal aspirate; PD = pancreatic duct. Numbers in parentheses are percents.

Puig et al found mt-K-ras in pancreatic fine-needle aspirates in 36/45 (80%) of samples analyzed by continuous-enrichment for the mutant allele by enzymatic digestion PCR, apparently without a single false positive (Puig et al. 2000). Strum et al found that 24/38 (63%) patients were correctly diagnosed with PDAC by analysis of endo-biliary brushings for mt-K-ras, compared to 8/38 (21%) cases using conventional cytology (Sturm et al. 1999). Interestingly, mt-K-ras was found in 8/74 (11%) patients with benign disease with no progression to malignant disease after a mean follow up of 30 months (Sturm et al. 1999). Tada et al found that >1% of the total DNA from pancreatic juice was mt-K-ras in 8/15 (53%) of patients with PDAC, 3/3 patients with an intraductal papillary neoplasm and 2/19 (11%) patients with benign disease (Tada et al. 1998).

Free soluble DNA in the circulation is greatly increased in the presence of malignancy (<25ng/ ml in healthy subjects) (Shapiro et al. 1983). Yamada et al detected mt-K-ras in the blood of 9/15 (60%) patients with PDAC (Yamada et al. 1998). Theodor et al found mt-K-ras in the serum of 14/20 (70%) patients with PDAC but none in six patients with chronic pancreatitis or in five normal subjects (Theodor et al. 1999).

Using cloning technology, Caldas et al found mt-K-ras in the stool of 6/11 (55%) patients with PDAC, which correlated with the mutations found in the primary tumour (Caldas et al. 1994). Using selective hybridisation, Wenger et al found mt-K-ras in the

stool of only 7/36 (19%) patients with PDAC compared to 28/36 (78%) of the tumours (Wenger et al. 1999). In the same study, 1/5 patients with chronic pancreatitis had mt-K-ras compared to two cases with mutations in the pancreatic tissue (Wenger et al. 1999).

From these studies we can conclude that mt-K-ras is an early and probably essential early event in the pathogenesis of the great majority, if not all, PDACs. It suffers from a poor specificity however and so the question arises that if it were to be used in a screening programme, exactly how this would be done.

The main focus of this thesis will be on the analysis of K-ras in high risk patients as will be discussed in more detail in Chapter 4.

TP53 Tumour Suppressor Gene

The TP53 gene is mutated in 50-77% of pancreatic cancers and is altered in ~50% of malignancies of all types (Rozenblum et al. 1997). In pancreatic cancer there is a mutational hotspot located on codon 273 but mutations are found throughout the coding sequence, particularly in exons 5-8. Mutant p53 protein has a longer half-life than wild-type (wt) p53 protein and the accumulation in cells can be detected by immunohistochemistry (Table 12).

Heinmoller et al found alterations in at least one of three main tumour suppressor genes (TP53, CDKN2A and SMAD4) in 165 (60%) of 277 pancreatic intraductal lesions including some cases with nuclear atypia (Heinmoller et al. 2000). Loss of

heterozygosity (LOH) of TP53 tended to occur later than for either CDKN2A or SMAD4, perhaps reflecting the dominant nature of many p53 mutations. Immunohistochemistry was positive for p53 in 81% of tumours with LOH compared to only in 38% of pancreatic intraductal lesions with TP53 LOH (Heinmoller et al. 2000).

Ruggeri et al found 71/13 (56%) PDAC tissue specimens staining positive with p53 antibodies compared to 0/16 of normal pancreata (Ruggeri et al. 1997). Coppola et al found positive p53 staining in 20/42 (48%) patients with pancreatic carcinoma and positive correlation with tumour grade but not with stage or survival (Dergham et al. 1997; Coppola et al. 1998). Kawesha et al found positive IHC staining in 64/157 PDACs but this was of no prognostic value and did not correlate with any biological or histological variable. As already mentioned above Dergham et al found that the incidence of p53 over-expression was significantly lower in patients with a family history of cancer (40%) compared to those without (72%) (Dergham et al. 1997).

Gansauge et al analyzed 80 tissue samples from patients with chronic pancreatitis for alterations of TP53 and K-ras (Gansauge et al. 1998). There were no cases of mt-K-ras (codon 12) detected by RFLP but there were eight alterations in TP53 (two in exon 5, four in exon 6, two in exon 7) identified by SSCP. DNA sequencing revealed one deletion of 21 amino acids (exon 5), four polymorphisms in exon 6 with no change in the amino acid sequence, one point mutation in exon 5 and two point mutations located in the intron between exons 6 and 7. The relevance of the findings is unclear and it should be noted in particular that molecular analysis was undertaken on whole (not

micro-dissected) specimens. In contrast Luttges et al never found mt-K-ras in 19/429 (4.4%) microdissected lesions from 8/30 patients with chronic pancreatitis but not a single instance of p53 immunopositivity (Luttges et al. 2000).

Yamaguchi et al identified p53 mutations in pancreatic juice using SSCP in 11/26 (42' cases with pancreatic cancer but not in cases with a mucin-producing adenoma and r in patients with chronic pancreatitis (Yamaguchi et al. 1999). Immunocytology with p53 antibodies using cells from pancreatic duct brushings was shown by Iwao et al to detect 36/44 (82)% pancreatic carcinomas (Iwao et al. 1997; Iwao et al. 1998). Unfortunately, a similar analysis by Maacke et al that confirmed this finding, in that 10/15 (67%) patients with pancreatic carcinoma had p53 over-expression in pancreatic cells obtained endoscopically, also found p53 over-expression in 16/27 (59%) patients with chronic pancreatitis (Maacke et al. 1997). It is likely that p53 over-expression in the ductular cells of chronic pancreatitis represents increased p53 activity rather than a manifestation of TP53 mutations.

Laurent-Puig et al found the sensitivity and specificity of enzyme linked immunosorbent assay (ELISA) for the detection of p53 antibodies in the sera patients with pancreatic cancer was 8/29 (28%) and 32/33 (97%) respectively (Laurent-Puig et al. 1995). Suwa et al found that only 16/47 (34%) patients with metastasis from pancreatic carcinoma had detectable serum p53 protein but only in 7/57 (12%) patients with carcinoma but no metastasis (Suwa et al. 1997).

Thus, we can conclude that mt-TP53 is a feature of most invasive pancreatic cancers and can be detected in some cases in pre-neoplastic lesions. The number of mutations makes the detection of mt-TP53 from DNA in pancreatic juice difficult but the specificity for pancreatic cancer appears to be very high even in cases with concomitant chronic pancreatitis. The early diagnostic data using analysis of DNA from pancreatic juice look promising.

Table 12: Detection of p53 protein in diagnostic clinical samples and pancreatic tissue in cases of pancreatic ductal adenocarcinoma

Series	No. in series	Sample	Metho-dology	p53 mutation
Yamaguchi et al (Yamaguchi et al. 1999)	42 (26 PC, 16 CP)	PJ	SSCP	Sen 11/26 (42) Spec 0/16 (100)
Maacke et al (Maacke et al. 1997)	42 (15 PC, 27 CP)	PJ	IHC	Sen 10/15 (67) Spec 16/27 (59)
Iwao et al (Iwao et al. 1998)	74 (44 PC, 30 CP)	PD brushing	IHC	Sen 36/44 (82) Spec 30/30 (100)
Laurent-Puig et al (Laurent-Puig et al. 1995)	62 (29 PC, 33 Control)	Serum	ELISA	Sen 8/29 (28) Spec 32/33 (97)
Suwa et al (Suwa et al. 1997)	154 (104 PC, 35 Controls, 15 CP)	Serum	ELISA	Sen 23/104 (22) Spec 48/50 (96)
Scarpa et al (Scarpa et al. 1993)	34	Tissue	IHC	14/34 (41)
Pellegata et al (Pellegata et al. 1994)	35	Tissue	Sequencing	18/35 (51)
Dergham et al (Dergham et al. 1997)	76	Tissue	IHC	33/76 (43)
Kasuya et al (Kasuya et al. 1997)	70	Tissue	IHC	43/70 (61)
Suwa et al (Suwa et al. 1997)	61	Tissue	IHC	28/61 (46)
Rozenblum et al (Rozenblum et al. 1997)	41	Tissue	M/D	31/41 (76)
Ruggeri et al (Ruggeri et al. 1997)	136	Tissue	IHC	71/136 (56)
Copolla et al (Coppola et al. 1998)	42	Tissue	IHC	20/42 (48)
Tomaszewska et al (Tomaszewska et al. 1999)	18	Tissue	IHC	18/18 (100)
Kawesha et al (Kawesha et al. 2000)	157	Tissue	IHC	64/157 (41)

PC = pancreatic cancer; CP = chronic pancreatitis; Sen = sensitivity; Spec = specificity; ELISA = enzyme linked immunosorbent assay; IHC = immunohistochemistry; SSCP = single-strand confirmatory polymorphism; M/D = microdissection and sequencing; PJ = pancreatic juice; PD = pancreatic duct. Numbers in parentheses are percents

CDKN2A (P16, MST1) Tumour Suppressor Gene

Loss of function of the CDKN2A (MST1) tumour suppressor gene occurs in ~85-98% of cases pancreatic cancer by homozygous deletion, intragenic mutation and transcriptional silencing via hypermethylation of CpG islands in the promoter regions of the gene (Caldas et al. 1994; Schutte et al. 1997; Kawesha et al. 2000). Caldas et al found CDKN2A homozygous deletions in 15/37 (41%) pancreatic carcinomas (27 xenografts and 10 cell lines) and sequence changes in 14 (38%) (Caldas et al. 1994; Muller et al. 1994). Schutte et al using methylation-specific PCR found inactivation of CDKN2A in 49/50 (98%) pancreatic carcinomas (propagated as xenografts or cell lines) (Schutte et al. 1997). Kawesha et al showed loss of expression the $p16^{INK4a}$ protein by immunohistochemistry in 136/157 (87%) PDAC paraffin-embedded tissue samples (Kawesha et al. 2000).

Moskaluk et al found that CDKN2A alterations were present in 3/9 pancreatic intra-ductal lesions from four pancreatic cancers shown to have CDKN2A alterations (Moskaluk et al. 1997). In the study by Heinmoller et al already referred to above, there was a tendency for LOH of CDKN2A to have occurred earlier in pancreatic intraductal lesions than for TP53or SMAD4 (Heinmoller et al. 2000).

Gerdes et al identified PanIN grade 1a lesions in 10/20 chronic pancreatitis lesions, of which four (but none of the 20 non-PanIN tissues) were negative for $p16^{INK4a}$ expression by immunohistochemistry; one case was shown to have a known CDKN2A polymorphism (c.442G >A; A148T) and two had inactivating hypermethylation of the

CDKN2A promoter (Gerdes et al. 2001). These studies suggest that inactivation of CDKN2A is an early event in the pathogenesis of pancreatic carcinoma but the significance of the alterations observed in chronic pancreatitis is not clear.

SMAD4 (DPC4) Tumour Suppressor Gene

The protein product (Smad4) of the tumour suppressor gene SMAD4 (located on 18q21.1) is involved in transmitting growth-suppressive signals by the transforming growth factor-ß (TGF-ß) pathway, which in turn regulates cell growth and differentiation through their ability to induce or repress transcription of various genes, including cell-cycle control genes (Grau et al. 1997). Receptor-regulated Smads (R-Smads 2 and 3) bind to a specific TGF-ß-related receptor at the cell surface, are phosphorylated on ligand stimulation, associate with a co-Smad (Smad4) and then translocate into the nucleus to regulate gene expression. TGF-ß requires Smad4 to inhibit pancreatic cell growth through upregulation of the cell cycle inhibitor $p21^{waf1}$ (Grau et al. 1997). Schwarte-Waldhoff et al postulated that part of the SMAD4 tumour suppression function is mediated via controlling the expression of vascular endothelial growth factor and thrombospondin-1 and imposing growth constraints to tumour cells by limited vascularization (Schwarte-Waldhoff et al. 2000).

SMAD4 is inactivated in ~50% of pancreatic cancers by homozygous deletion, LOH and intragenic mutation, (Hahn et al. 1996) compared to <10% in other tumour types (Schutte et al. 1996). Moskaluk et al found no mutations from analysis of the entire SMAD4 coding sequence of blood DNA from 25 individuals (11 kindreds) with a familial aggregation of pancreatic malignancy (Moskaluk et al. 1997).

Wilentz et al used DPC4 (SMAD4) IHC of formalin-fixed, paraffin-embedded tissue to study 188 PanINs in 40 pancreata, 38 (95%) of which also contained an infiltrating adenocarcinoma. All 82 flat (PanIN-1a), all 54 papillary (PanIN-1b) and all 23 atypical papillary (PanIN-2) intraductal lesions expressed DPC4 compared to only 20/29 (69%) of severely atypical lesions (PanIN-3 lesions, carcinomas in situ). The authors concluded that loss of DPC4 expression occurs biologically late in the progression of pancreatic cancer, at the stage of histologically recognizable carcinoma (Wilentz et al. 2000). In the study by Heinmoller et al, LOH of SMAD4 in pancreatic intraductal lesions, like TP53 tended, to occur later than LOH of CDKN2A (Heinmoller et al. 2000).

Fukushige et al studied the loss of 18q in cells obtained from pancreatic juice by ERCP using fluorescence in situ hybridisation (FISH) (Fukushige et al. 1998). Loss of 18q was found in 3/5 patients with pancreatic ductal adenocarcinoma compared to 0/11 (0%) of patients with chronic pancreatitis. Three tissue specimens of the patients with pancreatic ductal adenocarcinoma were also found to have 18q loss by FISH.

The European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) Secondary Screening Protocol in High Risk Patients

One of the major problems with screening for pancreatic cancer is that the best screening modalities are actually also the diagnostic modalities. This is because of the requirement for screening tests, as we have shown, to have a sensitivity of $\geq$ 95% and a specificity of 98% or 99.9% depending on whether the risk is 10-fold or 100-fold respectively.

Screening modalities should be relatively cheap and carry a minimum risk. On the other hand, the use of a test that has low cost and has little risk attached to it (such as EUS every year, say) but that has a sensitivity of $\leq$95% and/or a specificity of $\leq$98-99.9% will in reality be very costly and have an unacceptable risk because of the generation of a large amount of false positives.

Moreover the diagnosis of pancreatic cancer at an early stage is still very problematical, often requiring the use of multiple modalities in order to reach a reasonable level of diagnostic certainty. Even then the only way in which a certain diagnosis can be reached in ~5-10% of cases is by a partial pancreatectomy.

With these factors in mind a central objective of the EUROPAC protocol has been to modulate screening by reducing the interval for screening during the lesser risk periods and on the other hand, by focussing the intensity of diagnostic tests in higher risk situations (Howes et al. 2000). Above we have given the evidence demonstrating that the

diagnostic modalities of high quality dynamic-spiral CT, ERCP, EUS and probably MRI have a similar diagnostic accuracy in expert hands. The choice of which to use depends on local expertise. Because the diagnostic accuracy of any one modality is slightly less or at best just equivalent to that required (sensitivity $\geq$ 95%; specificity = 98-99.9%) it would be sensible to use a combination of modalities in the (as yet unproven) hope that the necessary marginal increase in accuracy would be achieved. A combination of EUS with CT performed annually would be one of several reasonable options.

The problem in HP, as opposed to FPC, is the reduced diagnostic accuracy of all of the modalities due to the background morphology of chronic pancreatitis in most of these patients. Thus reliance on the aforementioned approach (EUS with CT performed annually) is flawed.

An important tenet of the EUROPAC protocol is that diagnostic molecular methodology can be used to (i) identify higher and lesser periods of risk and (ii) ultimately be used for diagnosis. It is now clearly established that mt-K-ras arises in premalignant and even in normal cells. In pancreatic ductal cells with mt-K-ras the subsequent sequence of events is mutation/altered function of CDKN2A ($p16^{INK4a}$) in earlier pancreatic intraductal lesions followed by mutations in TP53, then SMAD4 in later pancreatic intraductal lesions and in early invasive carcinoma (Figure 7).

Mt-K-ras has been detected in pancreatic juice samples in with pancreatic cancer, chronic pancreatitis and in patients with normal pancreas glands. Whilst we have shown

that mt-K-ras does not have the specificity to diagnose pancreatic cancer, especially in the presence of chronic pancreatitis (Wong et al. 2000), we can also reasonably assume that PDAC is unlikely to arise in its absence. Thus, we hypothesize that we can use the detection of mt-K-ras to identify patients from families with HP who, for a period of time, are at higher (mt-K-ras present) or lower (mt-K-ras absent) risk. Furthermore, we hypothesize that a reasonable period before re-evaluation is required is three years in lower risk cases and one year in higher risk cases. Those at higher risk require immediate diagnostic evaluation (made easier if baseline diagnostic tests have been performed at previous visits). If the diagnosis of pancreatic cancer is not supported then re-testing in one year is reasonable. Since most patients are likely to be mt-K-ras negative and remain so, the total cost of the screening programme remains reasonable, despite the multiplicity of tests.

The use of molecular technology for diagnostic purposes is more problematical because of the large number of mutations involved and/or the complexity of the mechanisms of inactivation of the three main tumour suppressor genes. Nevertheless a clinical applicable test for TP53 mutation detection in DNA obtained from pancreatic juice has been developed by EUROPAC (Threadgold et al. 2000). This is a yeast functional assay for TP53 mutations previously developed to identify mutant TP53 at the transcriptional level (mRNA) (Inga et al. 1997; Flaman et al. 1998). The technique has been adapted to overcome the instability of RNA in pancreatic juice, by linking PCR products produced from exons 5-8 of the DNA in pancreatic juice. Following DNA extraction from the pancreatic juice and amplification, p53 exons 5-8 are introduced into a p53-expressing

plasmid and used to transfect reporter yeast. The technique involves an initial screen of clones that lack functional p53. PCR is used to screen out colonies that contain rearrangements or large deletions of p53, neither of which are typically found in pancreatic cancer. Finally clones are sequenced to eliminate mixed populations that would be ascribed to PCR errors and to identify the specific mutation. The sensitivity of this technique for the detection of mutant p53 in a background of wild type is as high as 1 in 3000 (Threadgold et al. 2000).

At the present time the identification of CDKN2A ($p16^{INK4a}$) and SMAD4 abnormalities in pancreatic juice is not a simple task. In approximately 60% of pancreatic cancers, there was CpG island methylation of at least 1 of 12 cancer-related markers including CDKN2A (Ueki et al. 2000). The aberrant CDKN2A methylation was found to occur at a rate of ~5-20% and it may be possible to apply a PCR-based method to detect for hypermethylation of CDKN2A and/or multiple loci in DNA from pancreatic juice samples.

Hypothesis, Aim and Evidence for EUROPAC Secondary Screening Protocol

The EUROPAC consortium believes that there is a progression of molecular markers in pancreatic juice that will allow risk stratification for the development of PDAC (Figure 8). These proceed from a K-ras mutation via a defined sequence of other mutations and markers through to a carcinoma identifiable with imaging. At some point along this preogression the development of carcinoma is inevitable and this point would be optimal for intervention.

Therefore, the aim of the EUROPAC protocol is to use imaging with CT and/or EUS, in combination with molecular analysis of pancreatic juice in high risk individuals, to detect PDAC.

The Department of Surgery in Liverpool have analysed pancreatic juice and produced data with regards to the determination of the risk of pancreatic cancer predicted/observed when compared alongside the results of molecular techniques (Yan et al. 2005).

	p53	RAS	p16 12%
Specificity v Pancreatitis	96 (86,99)	66(54,76)	90(70,97)
Specificity v Control	100(93-100)	79(67,87)	95(78,99)
Sensitivity	42(28,56)	54(42,68)	62(47,75)

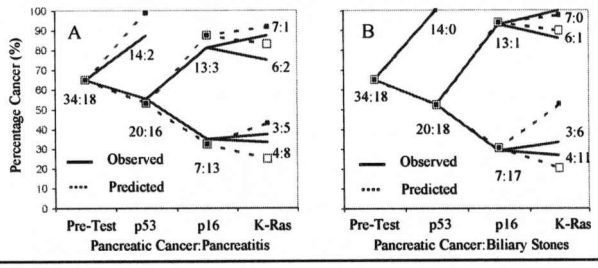


Figure 8: Graphs showing sensitivity and specificity of molecular techniques for detection of p53, Kras and p16 mutations (Yan et al. 2005)

Alongside the table are graphs showing the percentage of patients tested with all three modalities who had cancer given a positive or negative test (continuous lines). Graph A is comparing cancer and pancreatitis and graph B patients with biliary stones (controls) and cancer. In **A** 20 out of 36 patients with a negative p53 test (wild type p53) had cancer while 14 of 16 patients with a positive p53 test had cancer. The plot is continued including p16 and then K-Ras tests. A comparison is made (discontinuous line) with a prediction based on individual tests (97 p53 tests, 62 p16 tests, 124 K-Ras tests). The greater the independence of the tests the closer the match will be between predicted and observed curves. (Yan et al. 2005).

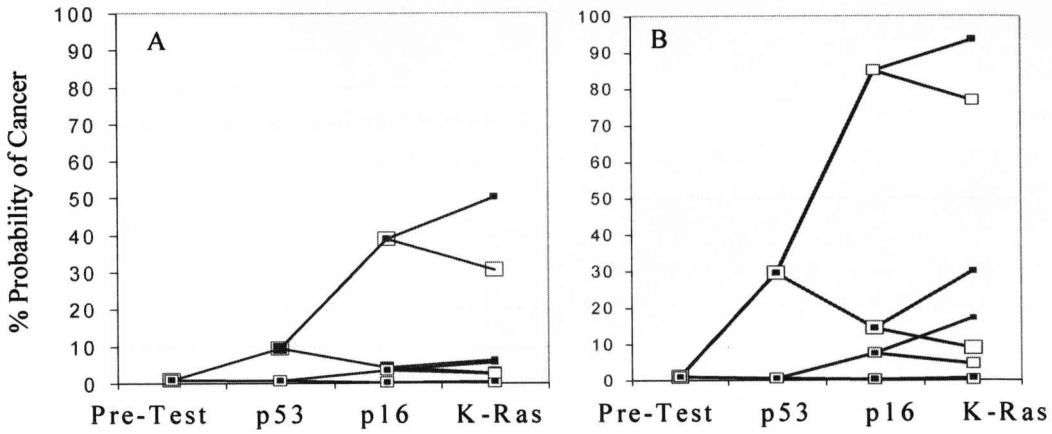


Figure 9: Graphs showing & probability cancer and series of molecular tests (Yan et al. 2005)

Using the same methodology, the percentage of individuals with cancer following positive or negative test results can be predicted assuming a level of pre-test prevalence equivalent to the probability of high-risk individuals developing cancer within a three year screening interval. The graphs shown below indicate the percentages given the series of molecular tests on a group of patients with pancreatitis (A) or no pancreatic disease (B). The proposed study will determine how accurate these predictions are for determining risk in HP (Graph A) or FPC (Graph B).

Inclusion criteria for Secondary Screening Recruitment

Hereditary Pancreatitis

Any patient with HP over the age of 40 years. Decision on the inclusion of a family as having HP is taken on the basis of a multidisciplinary group, including clinicians (I performed this role occasionally) and scientists (my supervisor WG often performed this role), a clinical geneticist was included when possible. Initial criteria for consideration of a pancreatitis family were as follows:

- Two relatives with chronic pancreatitis in two generations, in the absence of gallstones, a correlation with alcohol excess or other established non-genetic cause of pancreatitis.
- Individuals of any family who carry a predisposing mutation for HP (as described earlier).

The multidisciplinary committee considered the family tree and medical notes before classifying the family as having HP on the basis of autosomal dominance.

Familial Pancreatic Cancer

Individuals over 40 years of age from an established pancreatic cancer family, or occasional younger individuals where affected parents were younger than 50 or where affected siblings were younger than 40. As for HP a multidisciplinary committee decided on final inclusion of a family as having FPC. The criteria for consideration of a family were:

- Two first degree relatives with pancreatic ductal adenocarcinoma; and/or three or more relatives with pancreatic adenocarcinoma and/or

- Families with a causative gene linked to pancreatic cancer (e.g. BRCA2 or as yet undiscovered genes).

The multidisciplinary committee considered the family tree, medical notes and cancer registry reports before classifying the family as having FPC on the basis of autosomal dominance.

Individuals from these families will only be considered if they have a first degree relative with pancreatic cancer, or second degree if there is additional evidence that the individual is a carrier (e.g. identified BRCA2 mutation or a first degree relative with a descendent with cancer).

Exclusion criteria for Secondary Screening Recruitment

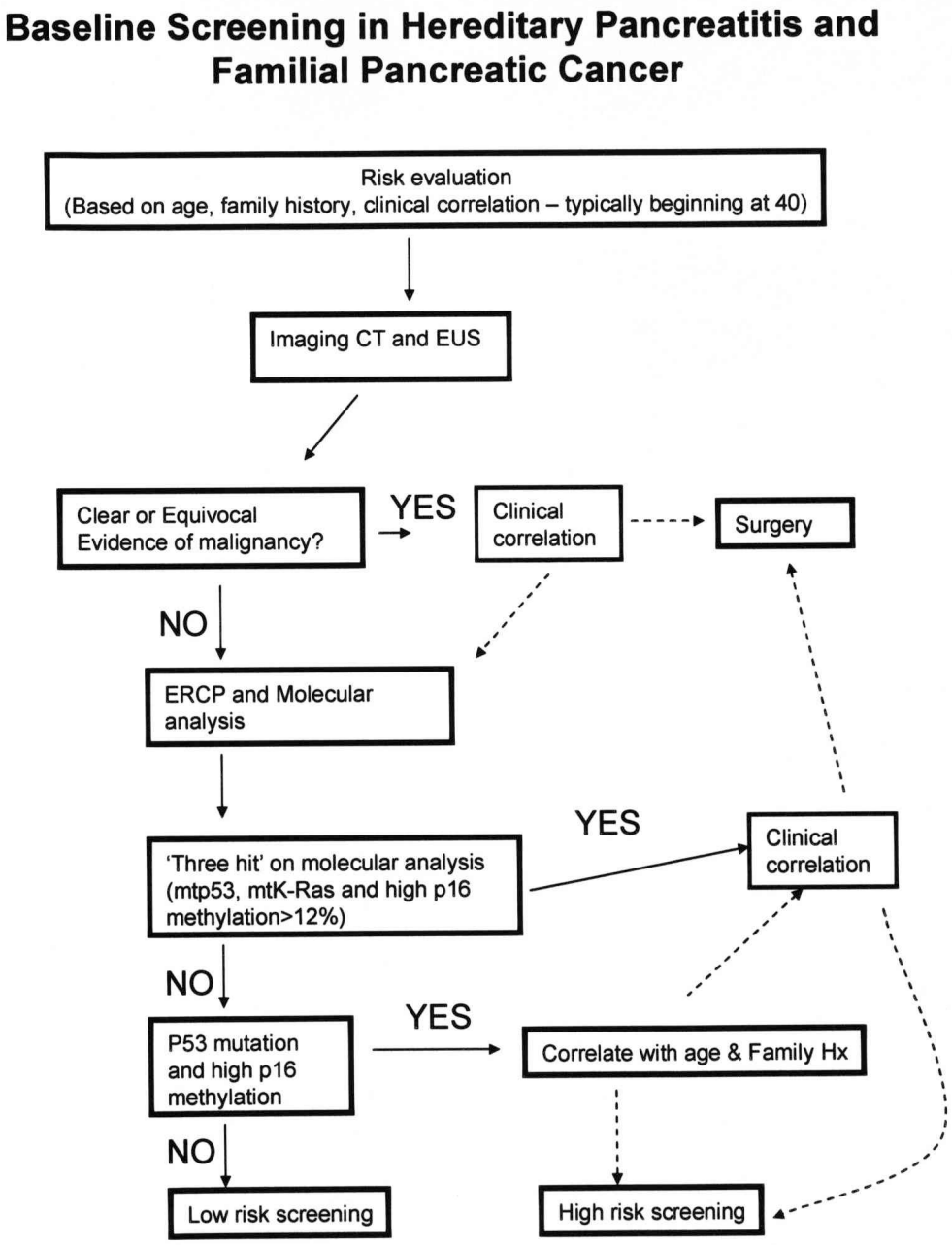
The following patients will not be recruited into the secondary screening programme for pancreatic cancer:

- Individuals under the age of 40 years with the exception of a small proportion of individuals from pancreatic cancer families where the family pedigree indicates a high risk of developing pancreatic cancer earlier than 40 years and a very small proportion of patients younger than the age of 40 years with Hereditary Pancreatitis who will undergo an ERCP as part of their routine clinical management.
- Any patient unable to give informed consent.
- Any woman able to bear a child but who has not taken appropriate contraceptive measures.

Secondary Screening Protocol

The EUROPAC consortium has produced flowcharts for the secondary screening of patients with Hereditary Pancreatits and individuals from Familial Pancreatric Cancer Families.

Figure 10: Baseline screening in Hereditary Pancreatits and Familial Pancreatric Cancer



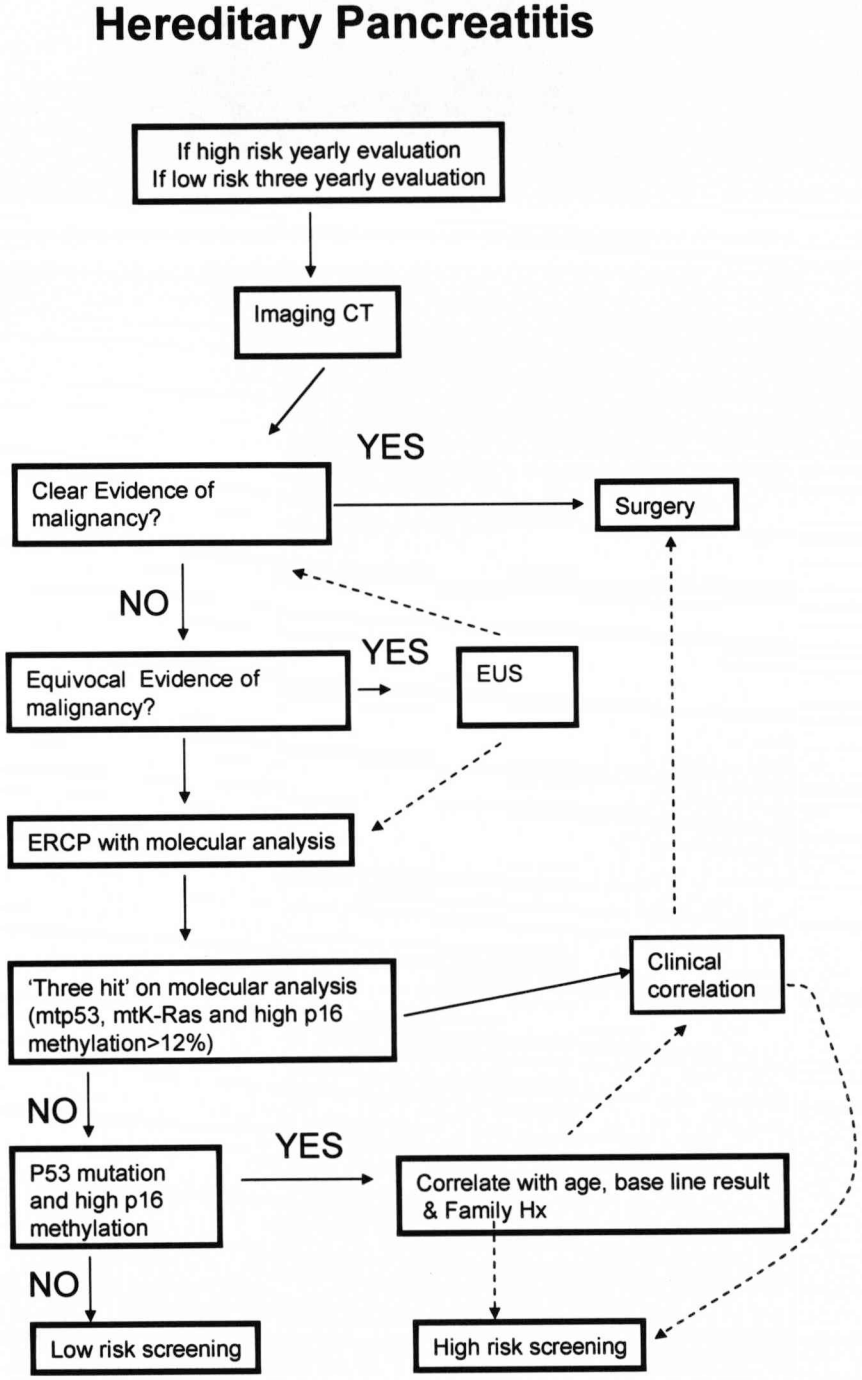


Figure 11: Secondary Screening protocol for patients with Hereditary Pancreatitis

Familial Pancreatic Cancer High Risk Screening

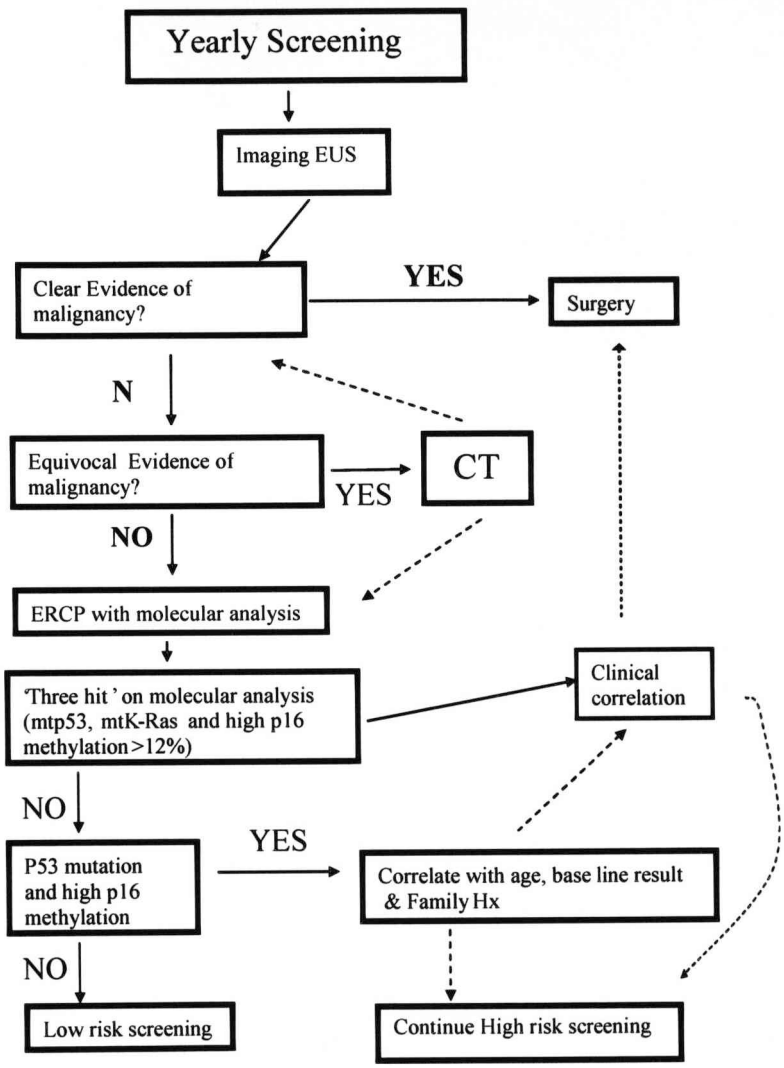


Figure 12: Secondary Screening protocol for Familial Pancreatic Cancer High Risk Group

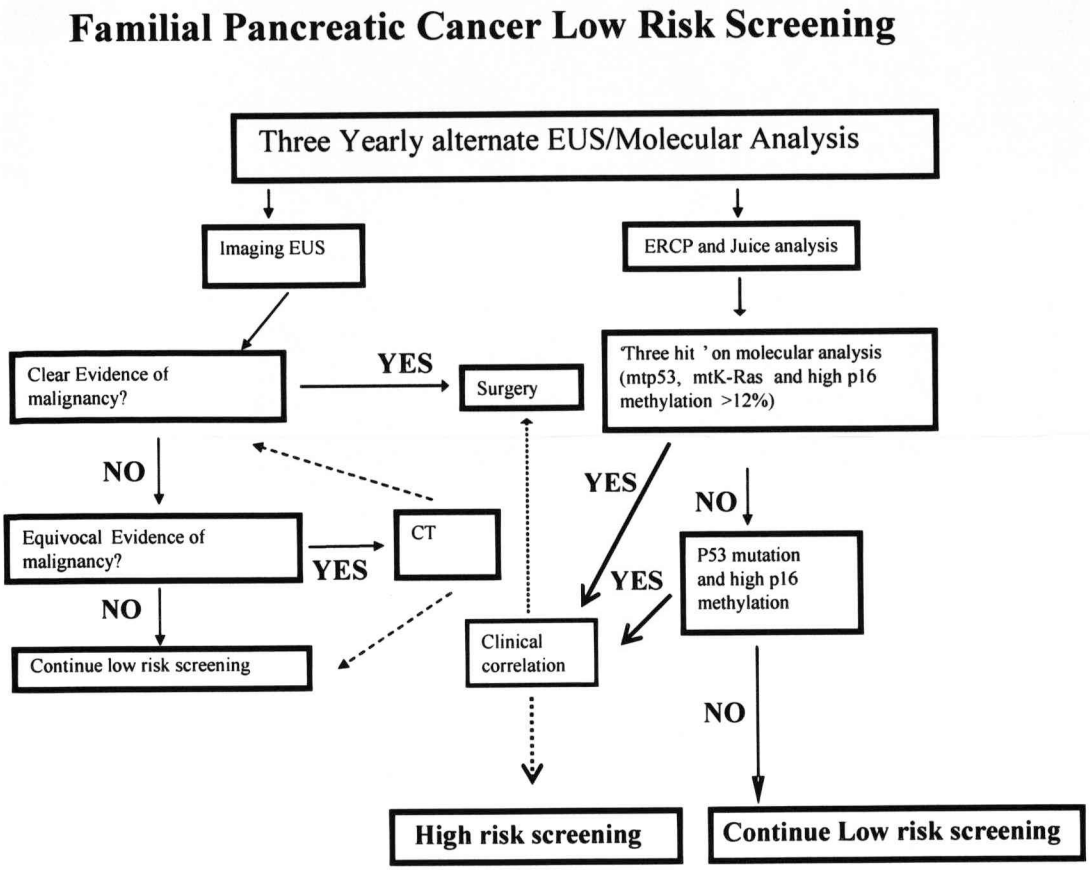


Figure 13: Secondary Screening protocol for Familial Pancreatic Cancer Low Risk Group

Discussion

Despite the rapid advances in the various imaging modalities and molecular biology techniques, our ability to detect early pancreatic ductal adenocarcinoma is still in its infancy. The identification of K-ras mutations, which is the most simple and sensitive of the available molecular approaches, is unsuitable for diagnosis in isolation, as it lacks specificity for cancer. The molecular diagnosis of pancreatic ductal adenocarcinoma must therefore involve the analysis of more than one molecular marker, such as TP3. As yet insufficient data have been obtained as to the accuracy of molecular analysis in the diagnosis of early pancreatic cancer. EUROPAC has made a decision to adopt and concentrate on a specific set of molecular examinations. The implications of these assays needs to be established by longitudinal clinical correlation and requires the integrated collaboration of all groups caring for patients with Hereditary Pancreatitis and other high risk groups which will require risk estimation of patients in high risk groups (this will not be discussed further in this thesis).

Aims and objectives

Based on the discussion in the previous sections and the difficulties we face in detecting PDAC at an early, potentially curable stage, the aim of this research is to stratify cancer risk in patients with HP based on germline genotype and development of novel molecular tests for somatic mutations in pancreatic juice.

Therefore, to achieve this aim, the incidence of an exon 4 polymorphism in the PRSS1 gene in patients with HP was analysed. A novel method was set up for this anlaysis. By studying this polymorphism and the distribution in the general population and in patients with HP with known and unknown gene mutation status, it was hoped to determine if the founder status of theser patients can be postulated and hence, determine the inheritance pattern for HP.

Secondly, the the incidence of p16 and MGMT methylation status in the DNA from pancreatic juice was analysed. Again, a novel method was developed. The analysis was to determine if the methylation status can be used as a marker/ indication for early changes in the genetics of pancreatic cells and therefore, in patients with high risk of pancreatic cancer this would be the first step to determine that these patients are suitable for more intensive screening.

Finally, the incidence of K-ras mutations from DNA from serum, pancreatic tissue and pancreatic juice from patients with pancreatic cancer, chronic pancreatitis and benign disease was studied. A novel method of detection for K-ras mutations was set up with adequate the sensitivity and specificity for potential future clinical used. Mutant K-ras sequences which were not available commercially had to be manufactured by cloning known mutant fragmented K-ras sequences into the pMOSBlue cloning system to produce sufficient amount of mutant K-ras for setting up the novel ARMS technique. Following successful optimization of the ARMS technique, the incidence of K-ras mutations from the clinical samples were analysed to determine if there is a pattern to the incidence and distribution of the type of mutation occurring in the different clinical samples. The aim was to be able to use this technique to detect mutant K-ras in sufficiently small amounts to identify patients who have acquired the K-ras mutations prior to developing overt malignancy, again, to offer more intensive surveillance or even possibly preventative surgery.

Chapter 2: Exon 4 Polymorphism PRSS1, Hereditary Pancreatitis and Pancreatic Cancer

Although it is clear that hereditary pancreatitis is associated with pancreatic cancer (Howes, Lerch et al. 2004), it is not clear whether each mutation type (R122H, N29I, A16V or those families negative for all PRSS1 mutations) has an equivalent cancer risk. Furthermore, it is clear that there is considerable heterogeneity within mutation groups in terms of disease severity (onset of pain, incidence of exocrine and endocrine failure etc.) (Howes, Lerch et al. 2004). Successful screening requires targeting to the highest risk individuals (Vitone et al. 2005), therefore the aim of this chapter was to examine founder effect within the mutation groups and relate this to cancer risk. To achieve this aim we investigated segregation of a single nucleotide polymorphism linked to PRSS1.

Hereditary pancreatitis is a rare genetic disease, characterized by recurrent attacks of acute pancreatitis (usually starting from childhood), progressing to chronic pancreatitis in the majority of cases and a 40% increased risk of pancreatic cancer in patients aged between 50 and 70. This disease pattern was first described by Comfort and Steinberg in 1952. This disease is transmitted in an autosomal dominant pattern, with incomplete penetrance of ~80%. The clinical features of the acute pancreatitis attacks and the resulting chronic pancreatitis is indistinguishable from other causes of acute and chronic pancreatitis.

One of the genes responsible for hereditary pancreatitis has been mapped to the long arm of chromosome 7, PRSS1. One of the mutations responsible for this disease is due to a single point mutation, a G to A transition, in the third exon of cationic trypsionogen. This mutation resulted in an arganine (R) (CGC) to histidine (H) (CAC) substitution at the 105 amino acid trypsinogen. This mutation was numbered R122H.

The proposed mechanism of action for the R122H mutation in hereditary pancreatitis is as follows- R117 functions as the initial site of hydrolysis of trypsin by trypsin itself, followed by further degradation of the molecule (internal hydrolysis sites are exposed). Substitution of histidine for arginine at residue 117 eliminates this initial hydrolysis site, rendering trypsinogen and/or trypsin resistant to autolysis and permanent inactivation. If trypsinogen is activated in the pancreas in quantities that exceed the inhibitory capacity of the trypsiongen-pancreatic secretory trypsin inhibitor complex (only able to inhibit 20% of potential trypsin activity), then trypsin could activate all of the other digestive proenzymes, initiate pancreatic autodigestion and cause pancreatitis. Thus, the cationic trypsinogen R122H mutation seems to cause pancreatitis by eliminating a key inhibitory mechanism.

A second mutation in cationic trypsinogen, involving a point mutation in exon 2, an A to T transversion was identified. This mutation resulted in an asparagine (N) (AAC) to isoleucine (I) (ATC) amino acid substitution at residue 14. This mutation was designated N29I. The clinical syndrome affecting patients with the R122H or N29I mutations are similar, although, the age of onset is slightly later and the clinical features less severe in

patients with the N21I mutation. The R122H and N29I mutations account for about 60% of mutations affecting the PRSS1 gene.

Other mutations of the PRSS1 genes have been reported but these are rare in occurance- these include the A16V, D22G, K23R and -28delTCC. Pfutzer et al (Pfutzer et al. 2002) have repotted on two further novel cationic trypsinogen mutations, N29T and R122C. The R122C mutation eliminates the arganine autolysis site as with R122H. The N29T mutation may enhance intrapancreatic trypsin activity.

Another gene known to contribute to causing hereditary pancreatitis involves the pancreatic secretory trypsin inhibitor (PSTI) gene. PSTI is a Kazal type 1 serine protease inhibitor (SPINK1) which is produced as a 79 amino acid precursor protein in acinar cells and is processed to become a 56 amino acid mature peptide that is secreted into pancreatic juice. PSTI is considered to be the first-line "safeguard" system that can inactivate about 20% of total trypsin activation if trypsinogen is accidentally converted to trypsin in acinar cells. Witt and Pfutzer (Pfutzer et al. 2000; Witt et al. 2000) reported on an A to G substitution in exon 3 that resulted in an Asparagine (AAT) to Serine (AGT) transition at amino acid 34 of the PSTI protein, N34S. Another mutation repotted in the PSTI gene involves a C to T substitution in exon 4 that resulted in an Arginine (CGC) to Cysteine (TGC) transition in amino acid residue 67, R67C. A list of the muations involved in HP in shown in Table 2.

Materials

New England BIOLABS$_{INC}$, UK

1. Fok1 Enzyme (Cat. No 109S)

KODAX , UK

1. Polaroid film

PERKIN ELMER, USA

1. AmpliTaq Gold^{TM} (Cat. No N808-0241)

QIAGEN, USA

1. QIAamp^{R} DNA Mini Kit (Cat. No 51304)
2. DNA Mini Kit (Cat. No 51306)
3. QIAEX II kit (Cat. No 20021)

SIGMA- ADRICH, UK

1. Agarose (Cat. No 13,704-9)
2. Ethidium bromide (Cat. No 56,512-1)

EQUIPMENTS

1. Polaroid camera, Kodax, UK
2. GeneAmp PCR system 9700, PE Applied Biosystems, USA
3. Centrifuge Spectrafuge 16M, National Labnet Co, USA
4. Pipette, Gilsons, USA
5. Grant hot water bath, Grant Instruments, UK

ANALYSIS SOFTWARE

1. Statview, SAS Institute, USA
2. Microsoft Office 2003- Excel, USA

Methods

Recruitment of Participants

Forty nine normal population, 54 hereditary pancreatitis mutation negative and 51 mutation positive hereditary pancreatitis DNA samples were obtained from Dr. R Mountford, Department of Clinical Genetics, Liverpool Women's Hospital, Liverpool for this study.

Exon 4 Polymorphism Polymerase Chain Reaction

We developed the following protocol for detection of the polymorphism. AmpliTaq Gold^{TM} with 10x PCR Gold Buffer and $MgCl_2$ solution was used for the PCR reaction. The following conditions were found to be the most suitable after optimization experiments. The enzyme was activated at 94°C for 12 minutes, the reaction was subjected to 35 cycles of 94°C denaturation for 30 seconds, followed by annealing at 60°C for 30 seconds. This was followed by chain elongation for 1 minute.

Following amplification, 5 µl of the exon 4 PCR products was loaded on 3% agrose gel stained with ethidium bromide.

The primer sequences used were:

Primer name	Sequence
HP4SF	AGCCCCACCACCTTTTGAGTT
HP4OKUR	AAGCTCTCCTCACGTGCAGTA

Desalting and Concentrating DNA from Solution

The remainder of the amplified DNA, 45 µl was purified and concentrated using the QIAEX II kit. The final volume of the DNA was 18 µl.

Restriction Enzyme Digestion with Fok1

The purified DNA, 18 µl, was placed in a 1.5 ml eppendorf and 2 µl of 10x NEBuffer 4 was added. One unit of Fok1 enzyme will digest 1 µg of DNA in 1 hour at 37°C in a total reaction of 50ul. The appropriate amount of enzyme was added to the mix and incubated for 1 hour at 37°C. The enzyme was then inactivated at 65°C for 20 minutes. The digested DNA underwent 3% agrose gel electrophoresis and the result was recorded using Polaroid film.

Statistics

The statistical analysis was carried out using Statview and Microsoft Excel at the Department of Surgery and Oncology, University of Liverpool.

Statistical analysis of the distribution of Exon 4 polymorphism PRSS1 in the control and hereditary pancreatitis family samples were calculated using the Hardy-Weinberg equation.

The Statview software was used to derive the cumulative survival plot for mutation negative HP families. Differences in cancer survival between the D32 and D32D mutation negative HP families were analysed using the Logrank (Mantel-Cox), Breslow-Geham-Wilcoxon, Tarone-Ware, Peto-Peto-Wilcoxon and Harrington-Fleming tests. A $p < 0.001$ was considered to be of statistical significance.

Results

Patient Demographics

Forty-nine normal samples were analysed. Fifty-four mutation negative samples were analysed- these were derived from 38 families. Thirty-one families contributed to the 51 HP mutation positive samples- 21 families had the R122H mutation, 9 families had the N29I mutation and 1 family had the A16V mutation.

Sizes of PCR Products

The sizes (base pairs, bp) of the PCR products after Fok1 digestion are listed below.

Wild type	145 bp 295 bp 315 bp
D32D	130 bp 145 bp 182 bp 295 bp
Heterozygote	double band at approximately 300 bp and an intensity difference between the 145bp and 182bp bands

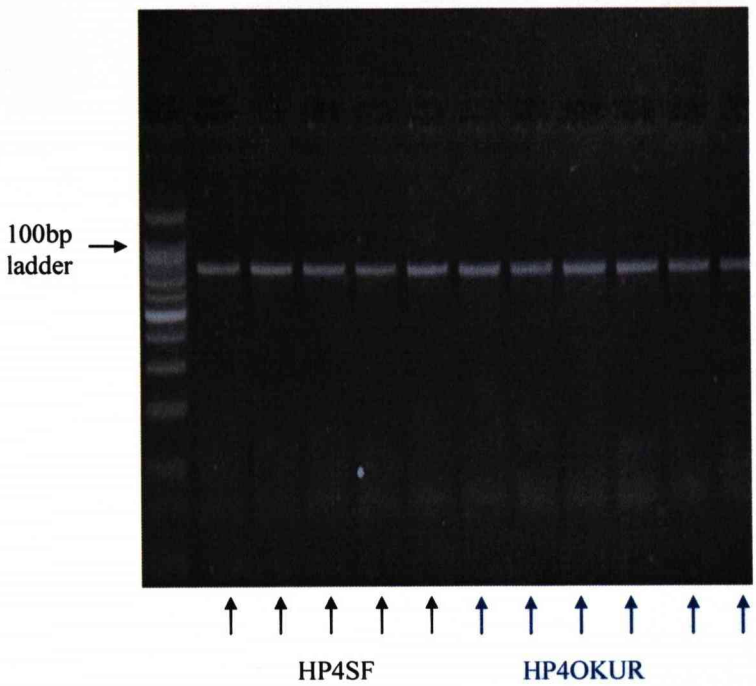


Figure 14: Agarose gel stained with ethidium bromide showing PCR amplified products with HP4SF and HP4OKUR

3% Agarose gel showing successful PCR with HP4SF (arrowed black) and HP4OKUR (arrowed blue) primers prior to digestion with FOK1 enzyme.

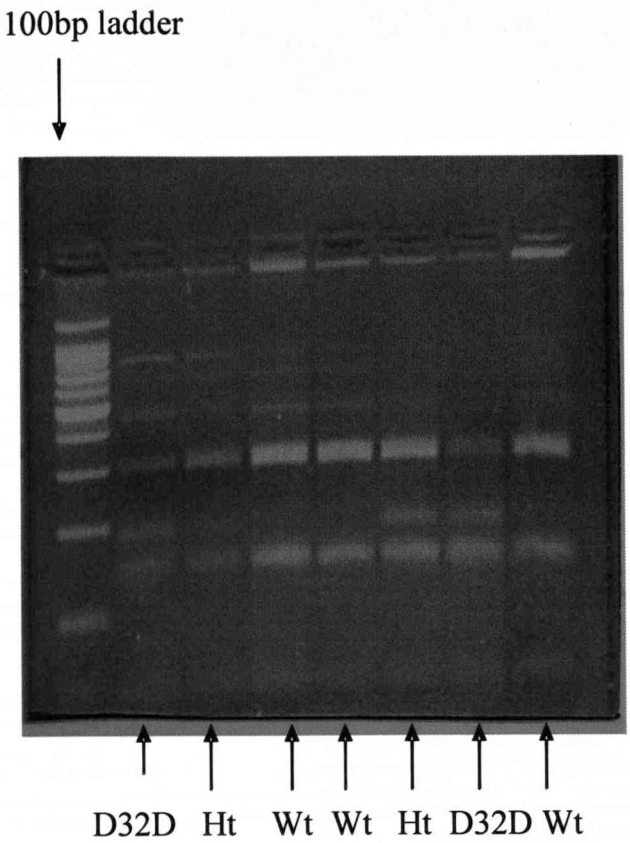


Figure 15: Agarose gel showing PCR products digested with Fok1 enzyme

3% agarose gel showing base pair sizes of PCR products after Fok1 digestion- Wt Wild type 145bp, 295bp, 315bp; D32D 130bp, 145bp, 182bp, 295bp; Ht Heterozygote double band at approximately 300bp, intensity difference at 145bp and 182bp.

Exon 4 Polymorphism Analysis in Control Samples

Before investigating the incidence of the different alleles in HP patients, we needed to investigate the allele frequencies in the general population. In this study, a rapid method based on restriction length polymorphism was developed to identify the polymorphism. Wild type sequences will give a pattern of 3 bands with our amplification and Fok1digestion at 315, 295 and 145 bp. D32D will give 4 bands of 182, 130, 295 and 145bp. Heterozygotes being identified by the presence of a double band at approximately 300 bp and an intensity difference between the 145 and 182bp bands.

Evolution is the sum total of the genetically inherited changes in the individuals who are the members of a population's gene pool. It is clear that the effects of evolution are felt by individuals, but it is the population as a whole that actually evolves. Evolution is simply a change in frequencies of alleles in the gene pool of a population.

In the early 20th century by Godfrey Hardy, an English mathematician, and Wilhelm Weinberg, a German physician developed independently the Hardy-Weinberg equation. Through mathematical modeling based on probability, they concluded in 1908 that gene pool frequencies are inherently stable but that evolution should be expected in all populations virtually all of the time. They resolved this apparent paradox by analyzing the net effects of potential evolutionary mechanisms.

Hardy, Weinberg, and the population geneticists who followed them came to understand that evolution will not occur in a population if seven conditions are met:

1. mutation is not occurring
2. natural selection is not occurring
3. the population is infinitely large
4. all members of the population breed
5. all mating is totally random
6. everyone produces the same number of offspring
7. there is no migration in or out of the population

These conditions are the absence of the things that can cause evolution. In other words, if no mechanisms of evolution are acting on a population, evolution will not occur--the gene pool frequencies will remain unchanged. However, since it is highly unlikely that any of these seven conditions, let alone all of them, will happen in the real world, evolution is the inevitable result.

Hardy and Weinberg went on to develop a simple equation that can be used to discover the probable genotype frequencies in a population and to track their changes from one generation to another. This has become known as the Hardy-Weinberg equilibrium equation. In this equation ($p^2 + 2pq + q^2 = 1$), p is defined as the frequency of the dominant allele and q as the frequency of the recessive allele for a trait controlled by a pair of alleles (A and a). In other words, p equals all of the alleles in individuals who are homozygous dominant (AA) and half of the alleles in people who are heterozygous (Aa) for this trait in a population. In mathematical terms, this is

$p = AA + ½Aa$

Likewise, q equals all of the alleles in individuals who are homozygous recessive (aa) and the other half of the alleles in people who are heterozygous (Aa).

$q = aa + ½Aa$

Because there are only two alleles in this case, the frequency of one plus the frequency of the other must equal 100%, which is to say

$p + q = 1$

Since this is logically true, then the following must also be correct:

$p = 1 - q$

There were only a few short steps from this knowledge for Hardy and Weinberg to realize that the chances of all possible combinations of alleles occurring randomly is

$(p + q)^2 = 1$

or more simply

$p^2 + 2pq + q^2 = 1$

In this equation, p^2 is the predicted frequency of homozygous dominant (AA) people in a population, 2pq is the predicted frequency of heterozygous (Aa) people, and q^2 is the predicted frequency of homozygous recessive (aa) ones.

From observations of phenotypes, it is usually only possible to know the frequency of homozygous recessive people, or q^2 in the equation, since they will not have the dominant trait. Those who express the trait in their phenotype could be either homozygous dominant (p^2) or heterozygous ($2pq$). The Hardy-Weinberg equation allows us to predict which ones they are. Since $p = 1 - q$ and q is known, it is possible to calculate p as well. Knowing p and q, it is a simple matter to plug these values into the Hardy-Weinberg equation ($p^2 + 2pq + q^2 = 1$). This then provides the predicted frequencies of all three genotypes for the selected trait within the population.

Exon 4 Polymorphism Analysis in Control Samples

Forty nine genomic DNA samples from patients with no known pancreatic disease were screened in this way. Of these samples 20/ 49 were heterozygous for D32D which is 41% Table 13.

Table 13: Distribution of Exon 4 polymorphism in Control Samples

	Homozygous High frequency (D32)	**Homozygous Low Frequency (D32D)**	**Heterozygous**	**Total**
Number	20	9	20	49
Percentage	41	18	41	100

	Actual	Ht calculated
p (allele frequency D32)	0.64 (= SQRT(0.41)	
q (allele frequency D32D)	0.43 (= SQRT(0.18)	
1-q	0.57 (1-0.43)	0.49 $=1-q^2-(1-q)^2$
1-p	0.36 (1-0.64)	0.46 $=1-p^2-(1-p)^2$

Table 14: Statistical Analysis of Distribution of Exon 4 polymorphism in Control Samples

Twenty nine of the forty nine (59%) patients had the D32D polymorphism. This is clearly inconsistent with the Hardy-Weinberg equilibrium, the sum of p and q are greater than one and the heterozygotes observed (41%) is inconsistent with the predicted proportion given the estimated proportion of homozygous D32 or on the basis of the proportion of homozygous D32D. This could be explained by a selective disadvantage of the heterozygote population (unlikely) or on the basis of at least two genetically distinct populations within our control group. The simplest explanation would be if 10 of the homozygous wild type controls came from a population with no D32D and the rest of the controls came from a population with 50% D32 and 50% D32D allele; in which case both populations would be in Hardy-Weinberg equilibrium assuming little or no contact between the populations.

Exon 4 Polymorphism Analysis in Hereditary Pancreatitis Family Samples

Family status/ Exon 4 status	R122H	N29I	A16V	Neg all
D32	9 (70%)	1 (25)	1 (100%)	15 (80%)
D32D	4 (30%)	3 (75%)		4 (20%)
Unknown	8	5	1	19

Table 15: Distribution of Exon 4 Polymorphism in HP Families

(The percentage of unknowns is biologically irrelevant. The important proportion is D32:D32D.

When analysing the results for the presence of the D32D polymorphism in the hereditary family sample group, we have to ignore the families with unknown D32D status.

For the 18 families with mutations analysed for the D32D polymorphism, the mutation occurs in both allelotypes. This is true for N29I and R122H, nothing can be said about A16V as only one family allowed haplotyping of the mutation with the polymorphism. This indicates the likelihood of multiple founders for the R122H families at least one in a D32 allelotype and at least one in D32D. The D32 population is more common so either a population linked to D32 has been more successful or there have been more founders in this group. The opposite is true for N29I, as in the general population D32D is less common than D32 this is more likely to indicate a limited number of founders –

the most successful of which was a D32D founder. Of course a single founder is possible in both cases with subsequent recombination.

In the hereditary pancreatitis families (19) with no known mutations, the distribution for the D32 to D32D families was 15 (79%) to 4 (21%). Compared to the control population, the mutation negative hereditary pancreatitis families had higher incidence of D32. It is probable that there is no linkage of pancreatitis with PRSS1 in these families, but we cannot rule out the possibility of an unknown mutation lying on this locus in at least some families. In which case it is likely the founder had a mutation on the D32 allele.

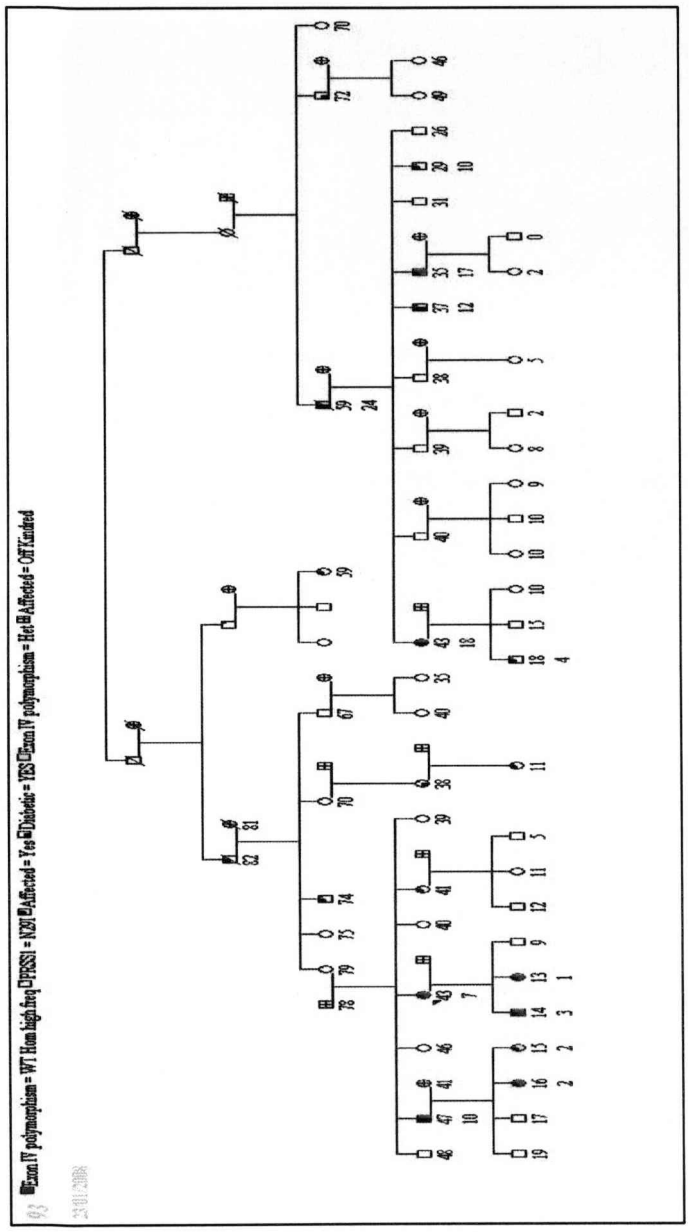


Figure 16: Example of Exon 4 polymorphism transmission in N29I HP family

In this example, the mutation occurred on an allele with D32 homozygous wild type and therefore the N29I is linked to D32.

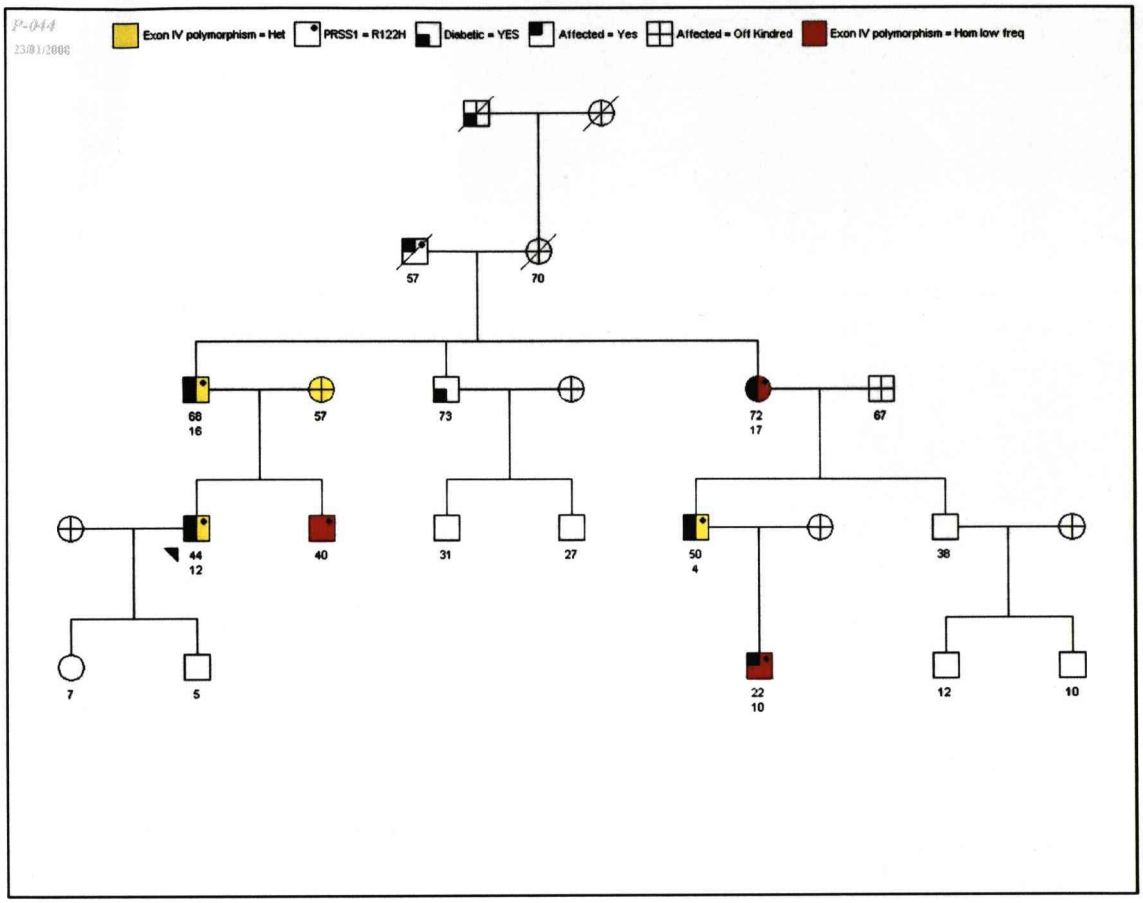


Figure 17: Example of Exon 4 polymorphism transmission in R122H HP family

In this example, the mutation occurred on an allele with D32D homozygous wild type and therefore the R122H is linked to D32D.

	Number Observed	Number of Cancer	Number Censored	% Censored
High Frequency (D32)	**49**	**3**	**46**	**93.878**
Low Frequency (D32D)	**18**	**0**	**18**	**100.000**
Untested	**215**	**12**	**203**	**94.419**
Total	**282**	**15**	**267**	**94.681**

Table 16: R122H Exon 4 polymorphism Hereditary Pancreatitis families Cancer Incidence

When we analysed the number of pancreatic cancer cases occurring in the R122H families with either the D32 or D32D allele profile, we found that there were 3 cancers occurring in the D32 (high frequency) group and no cancer occurring in the D32D (low frequency group). However, we are unable to determine the significance of this as the number analysed were too small for comment or stastistical analysis.

	Number Observed	Number of Cancer	Number Censored	% Censored
High Frequency (D32)	**17**	**0**	**17**	**100.000**
Low Frequency (D32D)	**17**	**2**	**15**	**88.235**
Untested	**90**	**4**	**86**	**95.556**
Total	**124**	**6**	**118**	**95.161**

Table 17: N29I Exon 4 polymorphism Hereditary Pancreatitis families Cancer Incidence

In the N29I families, there were 2 cancers observed in the low frequency group but again, the numbers are too small for comment or stastistical analysis.

	Number Observed	Number of Cancer	Number Censored	% Censored
High Frequency (D32)	**95**	**6**	**89**	**93.684**
Low Frequency (D32D)	**42**	**2**	**40**	**95.238**
Untested	**765**	**28**	**737**	**96.340**
Total	**902**	**36**	**866**	**96.009**

Table 18: Mutation Negative Exon 4 Polymorphism Hereditary Pancreatitis families Cancer Incidence

In the mutation negative hereditary pancreatitis families, there were 6 cancers observed in the exon 4 polymorphism (D32) group and 2 cancer observed in the low frequency (D32D) group.

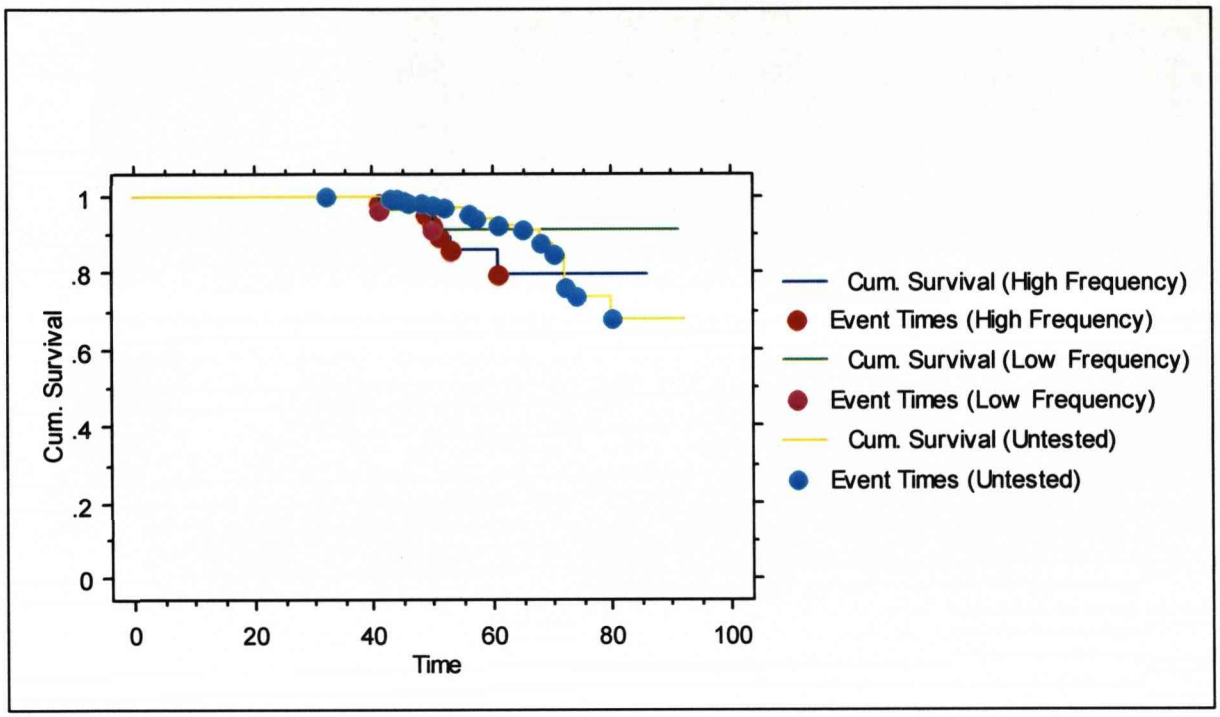


Figure 18: Graph of Kaplan-Mier Cumulative Survival plot for Mutation Negative Hereditary Pancreatitis Exon 4 Polymorphism families

The Kaplan-Mier survival plot shows that there is no significant difference in the cancer survival between the D32 and D32D mutation negative hereditary pancreatitis families.

	Chi-Square	DF	P-Value
Logrank (Mantel-Cox)	**1.017**	**2**	**0.6014**
Breslow-Gehan-Wilcoxon	**3.701**	**2**	**0.1572**
Tarone-Ware	**2.635**	**2**	**0.2678**
Peto-Peto-Wilcoxon	**1.372**	**2**	**0.5037**
Harrington-Fleming (rho = .5)	**1.140**	**2**	**0.5656**

Table 19: Stastistical analysis of survival data for Mutation Negative Hereditary Pancreatitis Exon 4 Polymorphism families

Stastistical analysis shows no significant difference in the cancer survival between the the D32 and D32D mutation negative hereditary pancreatitis families.

Discussion

The neutral polymorphism was noted on sequencing the DNA of patients with clinically defined hereditary pancreatitis but with no mutations in PRSS1. This polymorphism was the result of a single base change, cysteine to threonine, in codon 162, exon 4 of the PRSS1 gene not resulting in any change of the 32nd aspartate residue; we refer to this polymorphism as D32D. Of 49 patients examined 60% were heterozygous for the polymorphism. Those without the polymorphism we refer to as wild type. The polymorphism had previously been described (Nishimori et al. 1999) and although it was reported that the polymorphism appears in the general population no figures for frequency were presented. The high frequency we encountered with our PRSS1 wild type HP families suggested to us that the polymorphism might be in some way linked to the disease.

Chapter 3- Methylation

Cancer is a disease in which cells suffer from multiple genetic changes resulting in the abberant expression of many genes which in turn from tumour specific phenotypes. The methylation mediated gene silencing mechanism can contribute the tumour specific profile of gene expression, as a common alternative to genetic changes.

DNA methylation is one of several post-synthetic modifications that normal DNA goes through after each replication- one being the Cytosine-Guanine (CG) base pair is methylation. Since DNA replication is semi-conservative, one strand of the new DNA is already methylated and the other strand remains to be methylated by an enzyme called methyl transferase. Furthermore, the methyl transferases must recognize the appropriate base pair to be methylated as not all CG base pairs in DNA are methylated and this is to ensure that each piece of newly synthetized DNA is identical. In summary, the function of the methyl transferases are 1. the maintenance of the methylation status of genomes through DNA replication and 2. for the de novo DNA methylation in the early stages of development.

In humans, DNA is methylated only at cytosines located 5' to guanosine in the CpG dinucleotide. This modification has important regulatory effects on gene expression, especially when involving CpG-rich areas known as CpG islands, concentrated within short pieces of DNA segments. Gardiner-Garden and Frommer defined CpG islands as regions of DNA greater than 200bp with a G + C content >0.5 and an observed presence of CpG >0.6. Over 50% of the protein coding genes have at least one CpG island within

or near their promoters, expression of which are sensitive to the methylation status of such CpG islands (Baylin et al. 2000). Almost all gene –associated islands are protected from methylation on autosomal chromosomes, though extensive methylation of CpG islands has been associated with transcriptional inactivation of selected imprinted genes and the genes on inactivated X-chromosome of females. Abberant methylation of normally unmethylated CpG islands has been associated with transcriptional inactivation of a gene (Zingg et al. 1997).

P16

The p16 gene (also known as MTS-1, INK4a, CDKN2A), located on chromosome 9p21, encodes for a cyclin-dependent kinase inhibitor and is a G1-specific cell cycle regulatory gene. It is composed of three exons, which encode 156 amino acids. The gene is frequently inactivated in human cancers by 1. the genetic mechanism, that is, intragenic point mutations and small homozygous deletions and 2. the epigenetic mechanism, that is, methylation of the position 5 of cytosine promoter region (Nobori et al. 1994; Lo et al. 1996). Functional inactivation of p16 occurs when the second allele is either inactivated by a further inactivation event or the second allele is completely lost in a gene spanning deletion.

The exon 1 coding sequences of the p16 gene resides with the 5' CpG islands. This area is not methylated in most normal tissues but methylated in many human cancers.

DNA methylation plays an important role in the development, imprinting and aging. It has been reported that the methylation of estrogen receptor gene CpG island links aging in colonic mucosa (Issa et al. 1994).

Most CpG islands associated with genes are unmethylated in the germline and are often located within the promoter region of genes. These promoters are of variable size ranging from a few hundred bases to many thousand bases, methylation of the CpG sites within them can effectively, and heritably, lock genes in the 'off' condition. As few a 7% of methylation of CpG islands at the promoter sites can ensure gene quiescence (Hsieh 1997). Increased CpG island methylation is often accompanied by lowering in the overall level of DNA methylation and this might contribute to decreased chromosomal stability (Chen et al. 1998).

CpG islands can occur in the coding regions of genes and their positions relative to transcription sites can vary considerably- an example of a gene with an internal CpG island is the p16 gene. Methylation of a CpG island downstream of an active promoter in a mammalian gene does not block the formation of a transcript. CpG islands located downstream on transcription start sites show progressive de novo methylation changes, which are initiated in the aging process and reinforced during the formation of cancer.

In a study by Zhang et al. sixty paired samples of colorectal carcinoma and normal colonic mucosa were analysed for p16 methylation status. Statistical significant results showed positive correlation between p16 methylation and aging and no correlation

between p16 methylation and the clinical pathological parameters of the colorectal carcinoma patients (Zhang et al. 1999).

Yu et al found in patients with hepatocellular carcinoma, hypermethylation of the promoter CpG island of p16 may occur at a very late stage within the multiple stage processes of hepatocellular carcinogenesis (Yu et al. 2002).

MGMT

The gene encoding the DNA repair enayme O^6-methylguanine DNA methyltransferase (MGMT) has been found to be inactivated in several human cancers (Estellar et al. 1999). Alkylation of DNA at the O^6 position of guanine plays an important step in the formation of mutations in cancer, primarily due to the tendency of the O^6-methylguanine to pair with thymine during replication, resulting in a conversion of guanine-cytosien to adenine-thymine pairs in DNA. The O^6–alkylguanine-DNA adduct may cross-link with the opposite cytosine residues, blocking DNA replication. The MGMT protein protects cells from the toxicity of alkalyting agents, which frequently target the O^6 position of guanine. The MGMT protein rapidly reversed the formation of adducts at the O^6 position of guanine via transfer of the alkyl adduct to an active cysteine residue within the protein sequence, thereby preventing the formation of lethal cross-links and other mutagenic effects (Ludlum 1990; Pegg et al. 1995). Hence, the ability of a cell to withstand mutagenic and cytotoxic damage is directly related to the number of MGMT molecules it contains and to the rate of de novo synthesis of MGMT.

In human cancer, the MGMT gene is not commonly mutated, deleted or rearranged - the loss of MGMT function is most frequently due to epigenetic changes, specifically promoter region methylation and can play an important role in human neoplasia. Several studies have demonstrated hypermethylation of the MGMT CpG island as a cause of MGMT transcriptional silencing in cell lines defective in O^6 methylguanine repair (Qian et al. 1997; Watts et al. 1997; Danam et al. 1999; Estellar et al. 1999). Aberrant MGMT methylation has been associated with the loss of messenger RNA (mRNA) expression, lack of MGMT protein, and loss of enzymatic activity in neoplasia tissue (Estellar et al. 1999; Herfarth et al. 1999; Estellar et al. 2000).

Therefore, because hypermethylation of MGMT is associated with loss of mRNA expression and appears to be the only mechanism associated with loss of MGMT activitiy, MGMT loss of function can be studied by assessing promoter hypermethylation. This approach examines the epigenetic inactivation of the promoter itself rather than the loss of protein expression and enzyme activity.

The sequential acquisition of hypermethylation of tumour suppressor and mismatch repair genes at multiple gene promoter sites may also explain tumour progression from in-situ to invasive. House et al studied the methylation status of intraductal papillary mucinous neoplasms of the pancreas and found p16 genes in >50 of non-invasive and invasive of these lesions. MGMT repair genes were found to be methylated in 45% and 20% of invasive and non-invasive intraductal papillary mucinous neoplasms of the pancreas respectively. Hence, the detection of hypermethylation at multiple gene sites

may indicate the increased likelihood of invasive cellular behaviour and malignancy. This theory would be supported by our results of the methylation status in the pancreatic adenocarcinoma, chronic pancreatitis and control group analysed (House et al. 2003).

Methylation of tumour suppressor genes other than p16 and MGMT has also been identified in pancreatic cancer. Jansen et al found the tumour suppressor gene TSLC1 CpG island methylation to be present in pancreatic cancer and high grade PanIN-3 lesions. TSLC1 was reported to be methylated in 25/91 (27%) of pancreatic adenocarcinomas, 2/7 (29%) high-grade PanIN-3 lesions but not in low grade PanIN-1 (0/9), PanIN-2 (0/30) or normal pancreata (0/15) (Jensen et al. 2002).

Materials

CAMBREX, USA

1. NuSieve® 3:1 agarose (Cat. No 50090)

FERMENTAS, USA

1. Glycogen (Cat. No R0561)

NOVAGEN, USA

1. Pellet Paint™ Co-Precipitant (Cat. No 69049-3)

ONCOR, USA

1. CpG WIZ™ Amplification Kit p16 (Cat. No S7800)

PERKIN ELMER, USA

1. AmpliTaq Gold™ (Perkin Elmer, (Cat.No N808-0241)

PROMEGA, USA

1. Wizard® DNA Clean-Up System (Cat. No A7280)
2. 100bp DNA ladder (Cat. No G2101)

SIGMA-ALDRICH, UK

1. Agarose (Cat. No 13,704-9)
2. Ammonium acetate (Cat. No 37233-1)
3. Ethanol (Cat. No 60629-4)
4. Ethidium bromide (Cat. No 56,512-1)
5. Hydroquinone (Cat. No H1790-2)
6. Isopropanol (Cat. No 56395-5)
7. Sodium bisulfite (Cat. No S-8890)
8. Sodium hydroxide (Cat. No 48402-4)
9. Mineral oil (Cat. No M8691)

SOLUTIONS

1. 70% ethanol
2. 10mM hydroquinone
3. 80% Isopropanol
4. 2M NaOH
5. 3M NaOH
6. 10M Na_4Ac
7. 3M Sodium bisulfite
8. 2.5mM dNTP
9. 1x TAE

EQUIPMENTS

1. Polaroid camera, Kodax, UK
2. GeneAmp PCR system 9700, PE Applied Biosystems, USA
3. Centrifuge Spectrafuge 16M, National Labnet Co, USA
4. Pipette, Gilsons, USA
5. Grant hot water bath, Grant Instruments, UK

ANALYSIS SOFTWARE

1. Statview, SAS Institute, USA
2. Microsoft Office 2003- Excel, USA

Methods

DNA Sample Collection and Concentration

The DNA samples used for this study were obtained from the pancreatic juice collected, after informed consent, of patients undergoing ERCP under the EUROPAC study for molecular analysis. Approximately1500µl of pancreatic juice was aspirated from the pancreatic duct during ERCP or at the time of transaction of the pancreas during surgery and transported to the laboratory on ice. The first 1000µl was discarded from the ERCP samples, as this contained a high concentration of radiological contrast medium. Within two hours of collection the chilled pancreatic juice was transferred to sterile eppendorf tubes and spun at 4000g for ten minutes. The supernatant was aspirated and immediately stored at –80°C. The DNA had been extracted as in the QIAamp^{R}DNA Mini Kit protocol.

DNA Sample Extraction

The QIAamp^{R} DNA Mini Kit was used for DNA extraction from the pancreatic juice/ bile samples. For each DNA extraction, initially, 20 µl QIAGEN Protease (or proteinase K) was pipetted into the bottom of a 1.5 ml microcentrifuge tube. Up to 200 µl of pancreatic juice/ bile sample was added to the microcentrifuge tube. If the sample volume was less than 200 µl, the appropriate volume of PBS was added.

200 µl Buffer AL was added to the sample and mixed thoroughly to yield a homogeneous solution by pulse-vortexing for 15 s. The mixture was incubated at 56°C for 10 min. The mixture was briefly centrifuged to remove drops from the inside of the

lid. 200 µl ethanol (96–100%) was added to the sample, and mixed again by pulse-vortexing for 15 s. After mixing, the 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid.

The mixture was carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. The cap was closed, and the spin column centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

The QIAamp Mini spin column was carefully open and 500 µl Buffer AW1 was added without wetting the rim. The cap was closed and the spin column centrifuged at 6000 x g (8000 rpm) for 1 min.The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the collection tube containing the filtrate was discarded.

The QIAamp Mini spin column was carefully opened and 500 µl Buffer AW2 added without wetting the rim. The cap was closed and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The QIAamp Mini spin column was placed in a new 2 ml collection tube and the old collection tube with the filtrate was discarded. The spin column was centrifuged at full speed for 1 min.

The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp Mini spin column was carefully opened and 200 µl Buffer AE or distilled water was added. The mixture

was incubated at room temperature (15–25°C) for 1 min, and then centrifuged at 6000 x g (8000 rpm) for 1 min. A second elution step with a further 200 µl Buffer AE was performed to increase yields by up to 15%.

DNA Sample Concentration

The DNA in the pancreatic juice samples of patients with PDAC, chronic pancreatitis, and patients with biliary diseases were concentrated- 50 µl of the DNA samples were concentrated to a final volume of 20 µl (resuspended in PCR grade water) using Pellet Paint™ Co-Precipitant following the manufacturer's protocol.

Control DNA (wild type, unmethylated and methylated) for p16and MGMT methylation status was obtained from the CpG WIZ™ Amplification Kit p16.

DNA Methylation and Clean-Up

The patient DNA and control samples (except wild type) underwent bisulfite treatment following an adapted protocol from Frommer et al, as described below. Aberrant DNA methylation in the CpG Island of the genes by chemical modification would result in the unmethylated cytosine being converted to uracil, whereas the methylated xytosine remains unchanged.

For each sample, 2 µl of DNA was diluted into 50 µl with distilled water in a 1.5ml eppendorf and 5.5 µl of 2M NaOH added. This mixture was incubated at 37°C for 10 minutes. Thirty µl of 10mM hydroquinone* and 520 µl of 3M sodium bisulfite* were

added after the incubation period (*- these solutions must be freshly prepared). The mixture was thoroughly mixed, layered with mineral oil and incubated at 50°C for 20 hours.

The mineral oil was removed and the solution was cleaned-up using the Wizard® DNA Clean-Up System, following the manufacturer's protocol using a vacuum manifold. To the 50 µl final solution, 5.5 µl of 3M NaOH was added and incubated at room temperature for 5 minutes. One µl of glycogen carrier, 33 µl of 10M Na_4Ac and 270 µl of ethanol were added, incubated at -20°C for 30 minutes and spun in a microcentrifuge for 30 minutes. The supernatant was discarded; the precipitated DNA pellet washed with 70% ethanol and resuspended in 20 µl PCR grade water.

P16 and MGMT Methylation Status PCR

The primer sequences used for this study are as follows:

P16 W* forward 5' CAG AGG GTG GGG CGG ACC GC 3'
P16 W* reverse 5' CGG GCC GCG GCC GTG G 3'
P16 M* forward 5' TTA TTA GAG GGT GGG GCG GAT CGC 3'
P16 M* reverse 5' GAC CCC GAA CCG CGA CCG TAA 3'
P16 U* forward 5' TTA TTA GAG GGT GGG GTG GAT TGT 3'
P16 U* reverse 5' CAA CCC CAA ACC ACA ACC ATA A 3'
MGMT M* forward 5' TTT CGA CGT TCG TAG GTT TTC GC 3'
MGMT M* reverse 5' GCA CTC TTC CGA AAA CGA AAC G 3'
MGMT U* forward 5' TTT GTG TTT TGA TGT TTG TAG GTT TTT GT 3'
MGMT U* reverse 5' AAC TCC ACA CTC TTC CAA AAA AAC A 3'

* W= wild type; M= methylated; U= unmethylated status

The enzyme used for this PCR were AmpliTaq Gold™ with 10x PCR Gold Buffer and $MgCl_2$ solution.

The mastermix components for each 25 µl reaction are as listed below:

10x PCR Gold Buffer	2.5 µl
2.5 mM dNTP	2.5 µl
Primers (1pmol/µl)	1 µl
Taq	0.2 µl
Water	16.8 µl
Template DNA	2 µl

The enzyme is activated at 95°C for 12 minutes, and then the reaction was subjected to 35 cycles of amplification at 94°C for 45 seconds, followed by annealing at 59.5 °C for 45 seconds. This was followed by chain elongation at 72°C for 1 minute. The reaction was cooled to 4°C.

The PCR product (5 µl) was loaded onto a 5% gel (4 NuSieve®: 1 Agarose) with ethidium bromide (1 µl) and run in 1x TAE and visualised under UV illumination.

Statistical analysis of the data was undertaken using the Statview programme and data was analysed using the X^2 test. A $p < 0.001$ was considered to be of statistical significance.

The sizes (bp) of the PCR products are listed below.

Unmethylated p16	154 bp
Methylated p16	145 bp
Wild type p16	142 bp
Unmethylated MGMT	93 bp
Methylated MGMT	81 bp

Statistical analysis of Methylation status

The results from the experiments were analysed using the Statview and Microsoft Excel softwares at the Department of Surgery and Oncology, University of Liverpool.

Patients with a confirmed diagnosis of pancreatic ductal adenocarcinoma, chronic pancreatitis and benign biliary disease were taken as the reference groups. Correlation between p16 and GMT methylation and age, diagnosis and K-ras mutations categories were analysed using Statview Chi-square tests.

Cumulative onset of p16 and MGMT methylation with age and diagnosis was derived usong the Stativew Kaplan-Mier survival analyses. A Logrank $p < 0.001$ was considered to be of statistical significance.

Results

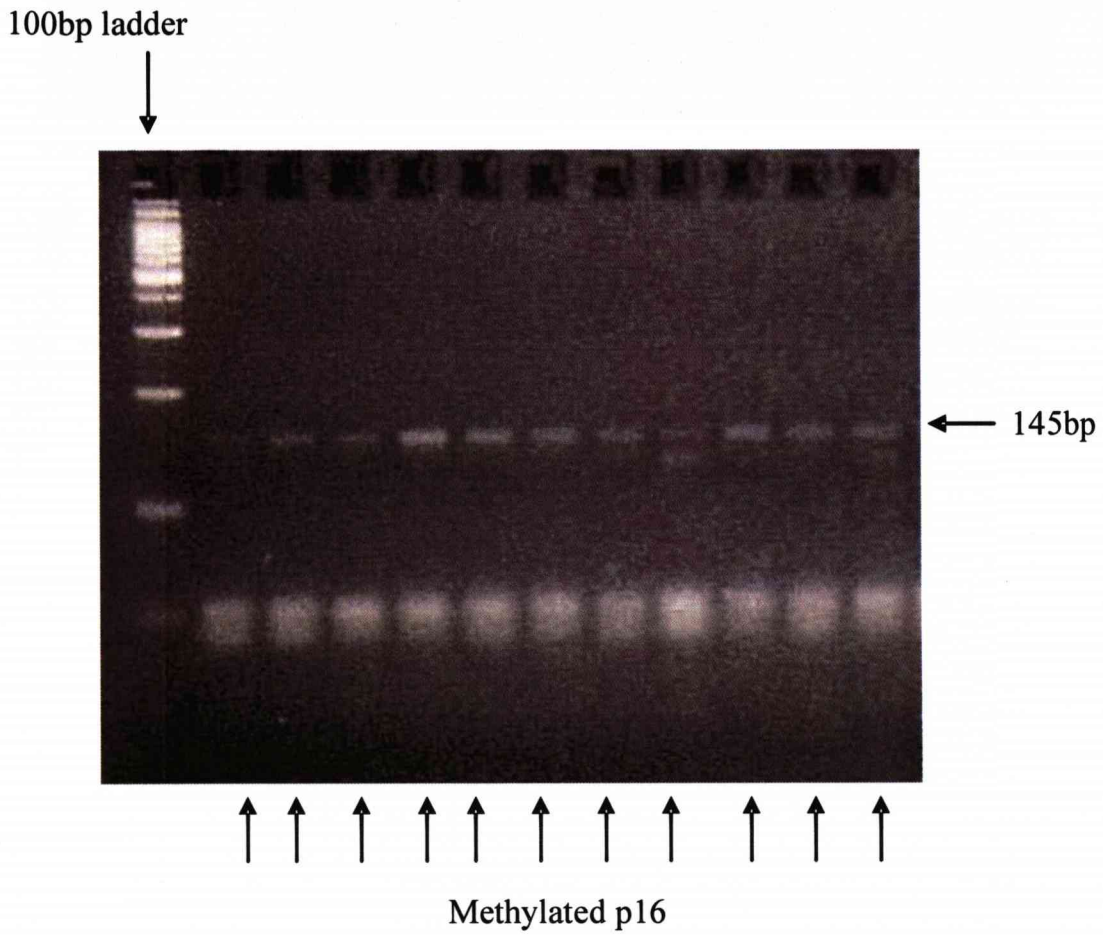


Figure 19: Gel picture of Methylated p16

Agarose gel showing amplified PCR products at 145bp for methylatd p16 DNA.

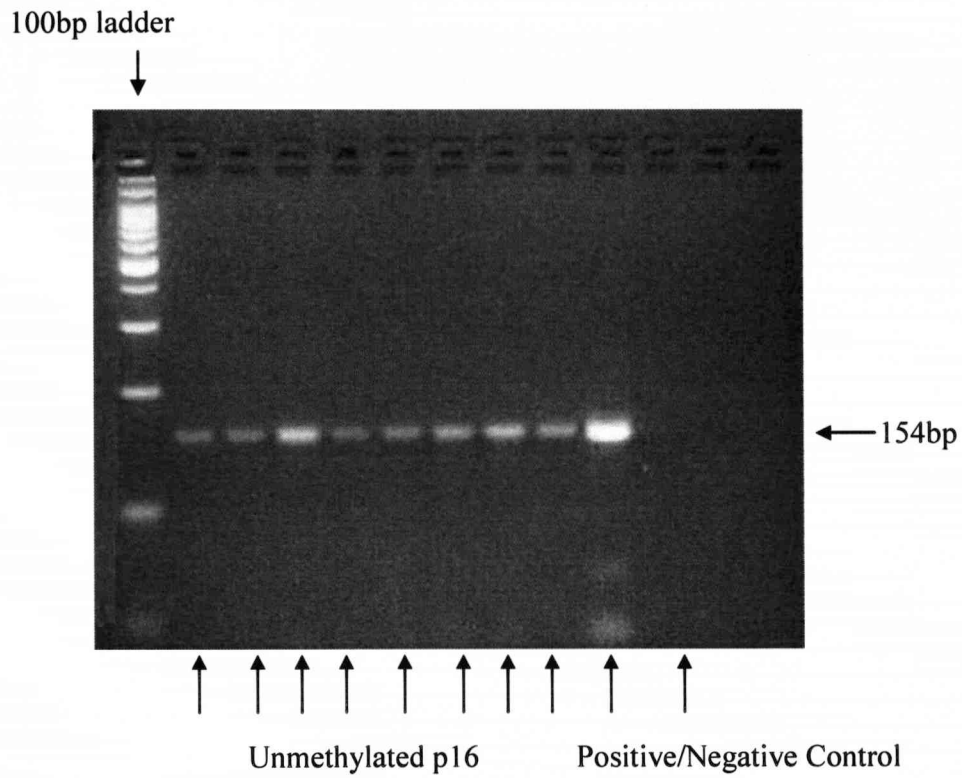


Figure 20: Gel picture of Unmethylated p16

Agarose gel showing amplified PCR products at 154bp for unmethylatd p16 DNA.

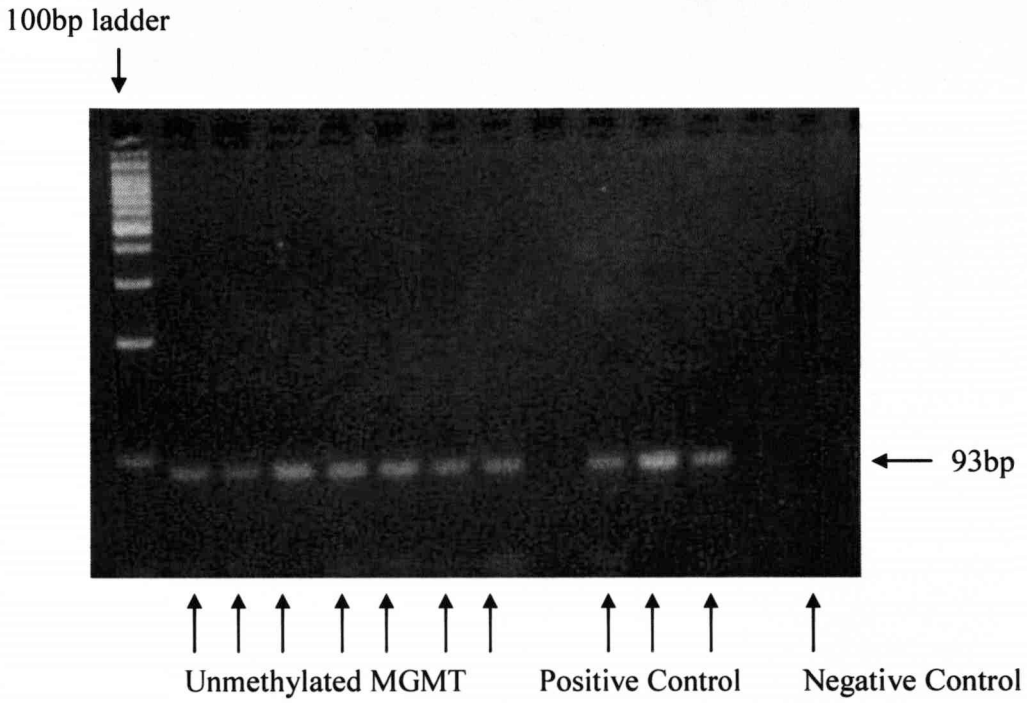


Figure 21: Gel picture of Unmethylated MGMT

Agarose gel showing amplified PCR products at 93bp for unmethylatd MGMT DNA.

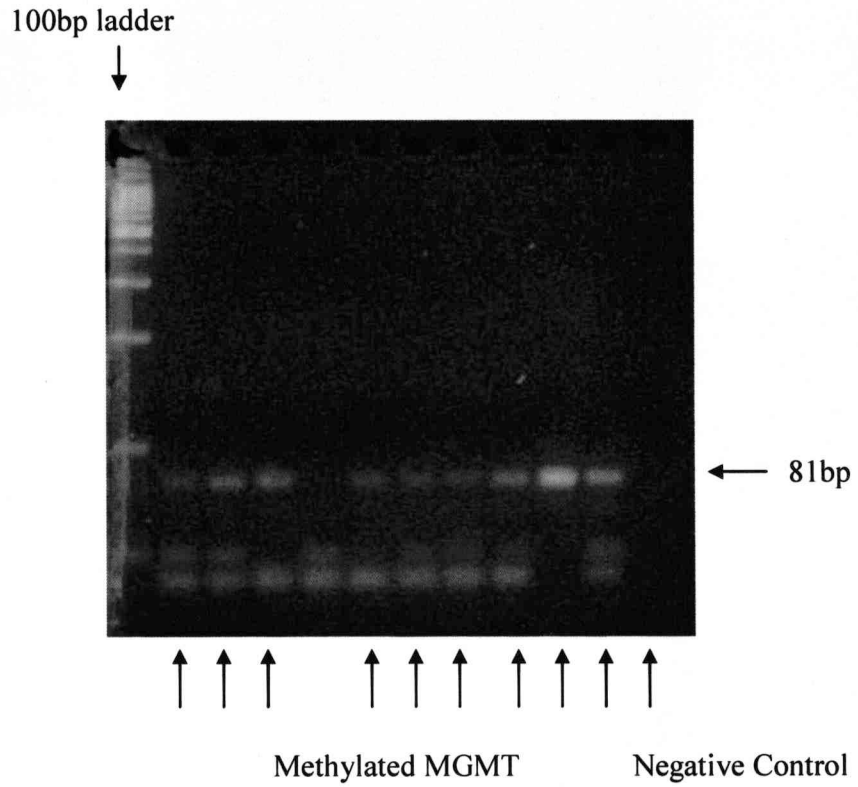


Figure 22: Gel picture of Methylated MGMT

Agarose gel showing amplified PCR products at 81bp for methylatd (as indicated) MGMT DNA.

Methylation Patient Demographics

The pancreatic juice DNA of 99 patients were analysed for p16 and MGMT methylation. The median age for these patients was 59 years. The cut-off point for the young-old category was 59 years. There were 51 patients in the young category and 48 patients in the old category. In this group, 30, 40, 25 and 4 pancreatic juice DNA samples were from PDAC, SpCP, Control and Cholangiocarcinoma patients respectively.

Correlation between p16 and MGMT methylation and age categories

In the old category, 36/48 (75%) patients were found to have methylated p16 compared to 28/51 (55%) in the young category. The expected frequency of p16 methylation in the young and old and young are 31/51 (61%) and 33/48 (69%) respectively. The number of patients found to have unmethylated p16 were 12/48 (25%) versus the expected 17/48 ((35%) in the old category and 23/51 (45%) versus the expected 18/51 (35%) in the young category. The differences in p16 methylation based on age category did not reach statistical significance. (Table 20) but we observed a trend for more methylation in older controls.

Table 20: Correlation between p16 Methylation in Young and Old Age Categories

	Observed frequency	**Expected frequency**
Methylated old	36	31
Unmethylated old	12	17
Methylated young	28	33
Unmethylated young	23	18

Chi-square p-value 0.0488; Median age 59 years old; Young-Old 59 years; Young 51 patients, Old 48 patients

Table 21: Correlation between MGMT in Young and Old Age Categories

	Observed frequency	Expected frequency
Methylated old	40	30
Unmethylated old	9	19
Methylated young	17	27
Unmethylated young	28	18

Chi-square p-value <0.0001; median age 59 years old; Young-Old 59 years; Young 45 patients, Old 49 patients

The analysis of MGMT methylation based on age category showed methylation in 40/49 (82%) in old and 17/45 (38%) in young patients versus the expected 30/49 (61%) in old and 27/45 (60%) in young patients. In the analysis for unmethylated MGMT, the observed frequency was 9/49 (18%) in old and 28/45 (62%) in young patients compared to the expected frequency of 19/49 (39%) in old and 18/45 (40%) in young patients respectively. The methylation status of MGMT based on the age category was statistical significance at a p-value <0.001 (Table 21). From the q analysis we observed more methylation in older patients.

Correlation between p16 and MGMT methylation with PDAC diagnosis and age categories

The pancreatic juice DNA of 31 patients with PDAC were analysed for p16 and MGMT methylation. The median age for the patients with PDAC was 60 years. The cut-off point for the young-old category was 60 years. There were 13 patients in the young category and 17 patients in the old category.

	Observed frequency	**Expected frequency**
Methylated old	17	17
Unmethylated old	0	0
Methylated young	13	13
Unmethylated young	0	0

Table 22: Correlation between p16 Methylation with PDAC in Young and Old Age Categories

Chi-square p-value not significant; median age 60 years old; Young-Old 60 years; Young 13 patients, Old 17 patients

In this group of patients all patients with PDAC had p16 methylation (Table 22), so nothing can be concluded about age and p16 methylation in cancer patients.

	Observed frequency	Expected frequency
Methylated old	16	15
Unmethylated old	1	2
Methylated young	10	11
Unmethylated young	3	2

Table 23: Correlation between MGMT with PDAC in Young and Old Age Categories

Chi-square p-value 0.1748; median age 60 years old; Young-Old 60 years; Young 13 patients, Old 17 patients

The analysis of MGMT methylation based on PDAC diagnosis and age category showed methylation in 16/17 (94%) in old and 10/13 (77%) in young patients versus the expected 15/17 (88%) in old and 11/13 (85%) in young patients. In the analysis for unmethylated MGMT, the observed frequency was 1/17 (6%) in old and 3/13 (23%) in young patients compared to the expected frequency of 2/17 (12%) in old and 2/13 (15%) in young patients. The methylation status of MGMT based on the age category did not reach statistical significance with a p-value 0.1748 (Table 23).

Correlation between p16 and MGMT methylation with Chronic Pancreatitis diagnosis and age categories

The pancreatic juice DNA of 40 patients with SpCP were analysed for p16 and MGMT methylation. The median age for the patients with PDAC was 48 years. The cut-off point for the young-old category was 50 years. There were 22 patients in the young category and 18 patients in the old category.

	Observed frequency	**Expected frequency**
Methylated old	7	7
Unmethylated old	11	11
Methylated young	8	8
Unmethylated young	14	14

Table 24: Correlation between p16 Methylation with Chronic Pancreatitis in Young and Old Age Categories

Chi-square p-value 0.296; median age 48 years old; Young-Old 50 years; Young 22 patients, Old 18 patients

In the old category, 7/18 (39%) of the patients were found to have methylated p16 compared to 8/22 (36%) in the young patients category and the number of patients found to have unmethylated p16 were 11/18 (61%) in the old category and 14/22 (64%) in the young category. The expected values for methylated and unmethylated p16 in this group of patients are the same as the observed frequencies. The methylation status of p16 in SpCP patients did not reach statistical significance with a p-value 0.296 (Table 24).

	Observed frequency	Expected frequency
Methylated old	1	1
Unmethylated old	15	15
Methylated young	2	2
Unmethylated young	15	15

Table 25: Correlation between MGMT with Chronic Pancreatitis in Young and Old Age Categories

Chi-square p-value 0.971; median age 48 years old; Young-Old 50 years; Young 17 patients, Old 16 patients

The analysis of MGMT methylation based on the chronic pancreatitis diagnosis and age category showed methylation in 1/16 (6%) in old and 2/17 (12%) in young patients. In the analysis for unmethylated MGMT, the observed frequency was 15/16 (94%) in old and 15/17 (88%). The expected frequency for MGMT methylation matched the observed frequency. The methylation status of MGMT based on the chronic pancreatitis diagnosis and age category did not reach statistical significance with a p-value 0.971 (Table 25).

Correlation between p16 and MGMT methylation with Control patients and age categories

The pancreatic juice DNA of 27 control patients were analysed for p16 and MGMT methylation. The median age for the patients with PDAC was 68 years. The cut-off point for the young-old category was 68 years. There were 14 patients in the young category and 13 patients in the old category.

	Observed frequency	**Expected frequency**
Methylated old	7	8
Unmethylated old	5	4
Methylated young	9	8
Unmethylated young	4	5

Table 26: Correlation between p16 Methylation with Control in Young and Old Age Categories

Chi-square p-value not significant; median age 68 years old; Young-Old **68 years**; Young 13 patients, Old 12 patients

P16 methylation results were available for 25 patients- 13 young and 12 old (young-old cut-off at 68 years based on median age). In the old category, 7/12 (58%) of the patients were found to have methylated p16 compared to 9/13 (70%) in the young patients category and the number of patients found to have unmethylated p16 were 5/12 (42%) in the old category and 4/13 (30%) in the young category. The expected values for methylated and unmethylated p16 in the old and young patient groups are 8/12 (67%), 4/12 (33%), 8/13 (62%) and 5/13 (38%) respectively. The methylation status of p16 in control patients did not reach statistical significance (Table 26). The results show a trend for methylation in the younger group of patients.

	Observed frequency	Expected frequency
Methylated old	13	12
Unmethylated old	5	6
Methylated young	3	4
Unmethylated young	4	3

Table 27: Correlation between p16 Methylation with Control in Young and Old Age Categories

Chi-square p-value not significant; median age 68 years old; Young-Old **60 years**; Young 7 patients, Old 18 patients

Analyses of the same data, altering the young-old cut-off to 60 years (for comparison with the PDAC group), p16 methylation results were available for 7 young and 18 old patients. The observed frequency for p16 methylation in the old and young patients were 13/18 (72%) and 3/7 (43%) compared to the expected values of 12/18 (67%) and 4/7 (57%) Table 27.

	Observed frequency	Expected frequency
Methylated old	11	12
Unmethylated old	2	1
Methylated young	13	12
Unmethylated young	1	2

Table 28: Correlation between MGMT with Control in Young and Old Age Categories

Chi-square p-value not significant; median age 68 years old; Young-Old 68 years; Young 14 patients, Old 13 patients

The analysis of MGMT methylation in young-old control patient categories showed methylation in 11/13 (85%) in old and 13/14 (93%) in young patients. In the analysis for unmethylated MGMT, the observed frequency was 2/14 (14%) in old and 1/14 (7%). The expected frequencies for MGMT methylation were 12/13 (92%) old and 12/14 (86%) in old and young patients respectively. The methylation status of MGMT analysed in control patients and age category did not reach statistical significance (Table 28).

Correlation between p16 and MGMT methylation and K-ras mutations in all patient groups

The pancreatic juice DNA of 99 patients were analysed for correlation between p16 methylation and K-ras mutations, age categories not taken into account. P16 methylation was found in 26/33 (79%) patients with mutant K-ras compared to 27/48 (56%) wild-type K-ras patients. The expected values for p16 methylation were 22/33 (67%) and 31/48 (65%) in mutant and wild-type K-ras patients respectively. The correlation of p16 methylation and K-ras mutation status did not reach statistical significance (Chi- square p-value 0.1112) (Table 29).

	Observed frequency	**Expected frequency**
Methylated/ K-Ras Mutant	26	22
Unmethylated/ K-Ras Mutant	7	11
Methylated/ K-Ras Wild type	27	31
Unmethylated/ K-Ras Wild type	21	17

Table 29: Correlation between p16 Methylation and K-ras mutations

Chi-square p-value 0.1112, total number of patients 99

The analysis of the correlation between MGMT methylation with K-ras mutational status yielded an observed frequency of MGMT methylation in 26/33 (79%) mutant K-ras and 26/46 (57%) wild-type K-ras patients. The expected methylation frequencies were calculated as 22/33 and 30/46 for mutant and wild-type K-ras patients respectively. The correlation between MGMT methylation and K-ras mutational status did not reach statistical significance (Chi-square p-value 0.0713) (Table 30).

	Observed frequency	**Expected frequency**
Methylated/ K-Ras Mutant	26	22
Unmethylated/ K-Ras Mutant	7	11
Methylated/ K-Ras Wild type	26	30
Unmethylated/ K-Ras Wild type	20	16

Table 30: Correlation between MGMT Methylation and K-ras mutations

Chi-square p-value 0.0713, total number of patients 79

Cumulative Onset of P16 and MGMT Methylation with Age

The analysis of the results using Kaplan-Mier survival analyses shows a methylation trend with increasing age when the patients were not divided into diagnosis subdivisions. This is true for p16 and MGMT methylation with non-significant Logrank p-values of 0.2213 (Figure 23) and 0.0062 (Figure 24) respectively.

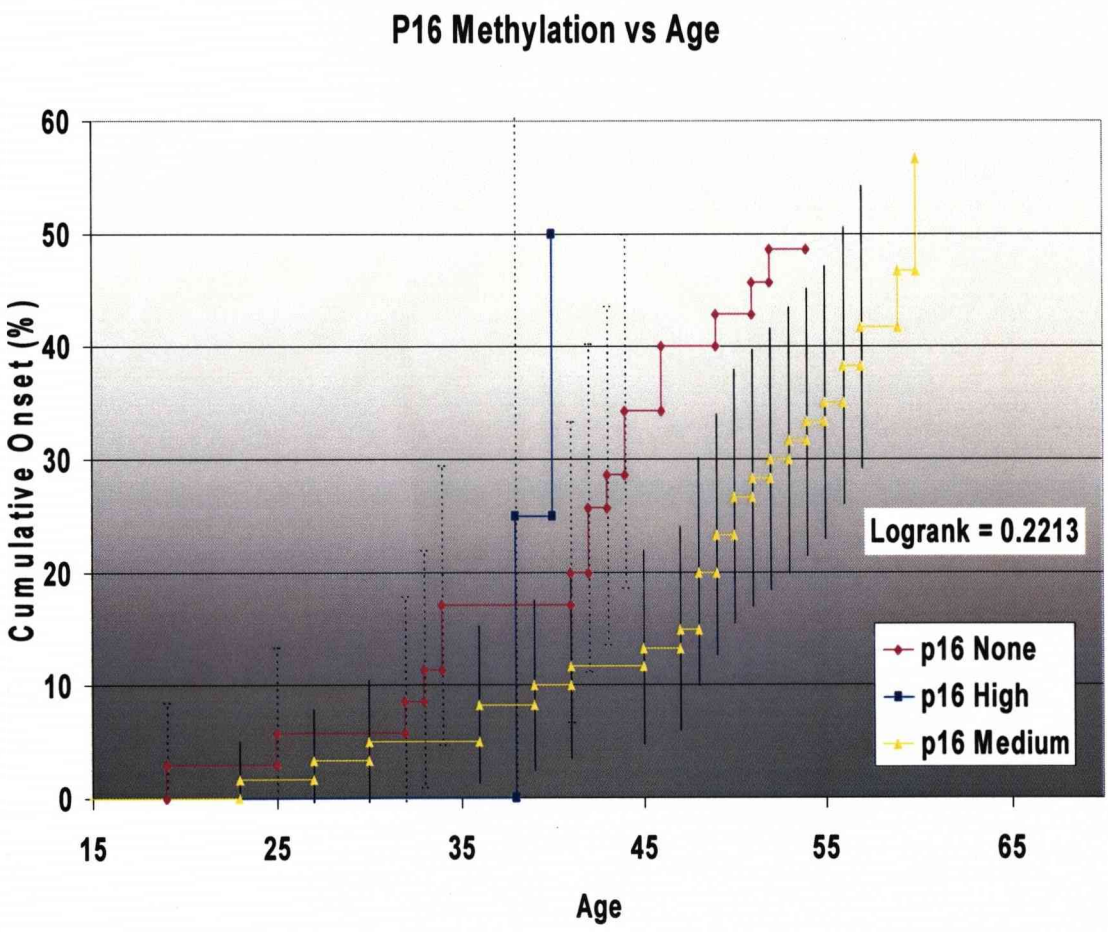


Figure 23: P16 Methylation vs Age

Cumulative analysis based on disease diagnosis again yielded graphs with trends of p16 and MGMT methylation occurring with increasing age. The general age by which p16 start to occurs appears to by around 30 years old in the Control, PDAC and Chronic Pancreatitis patients.

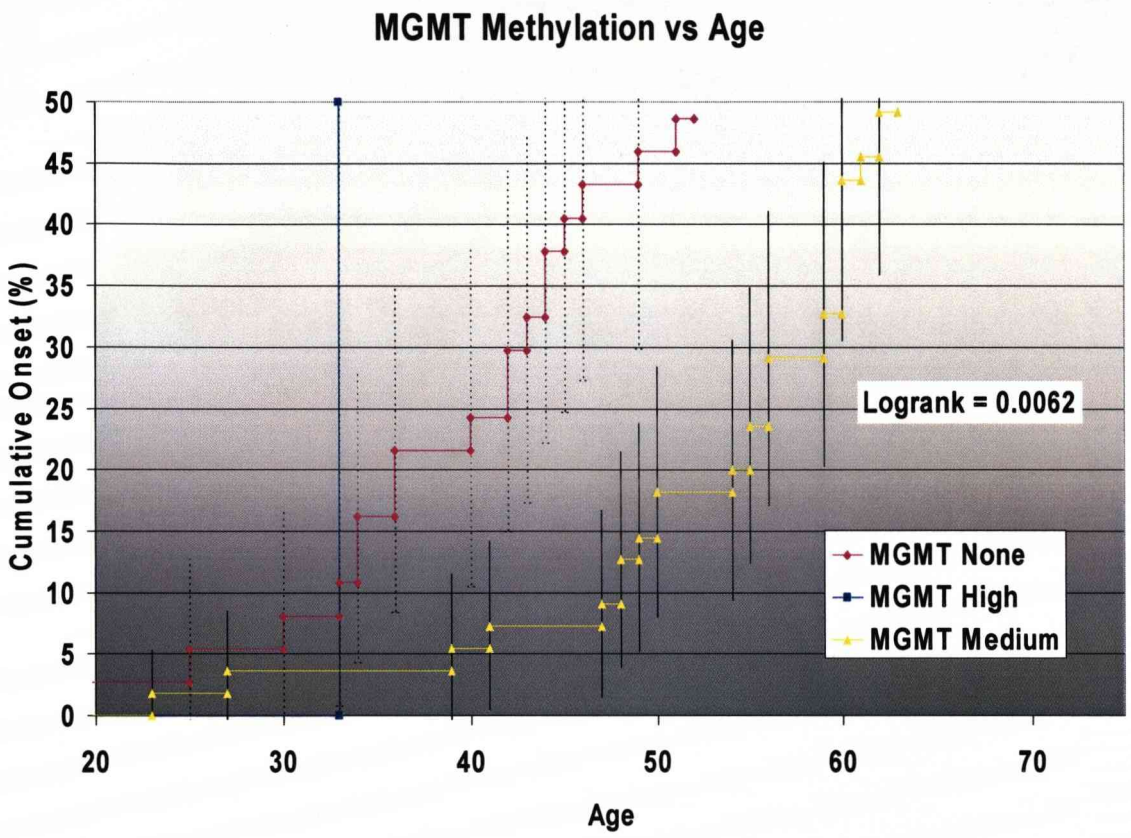
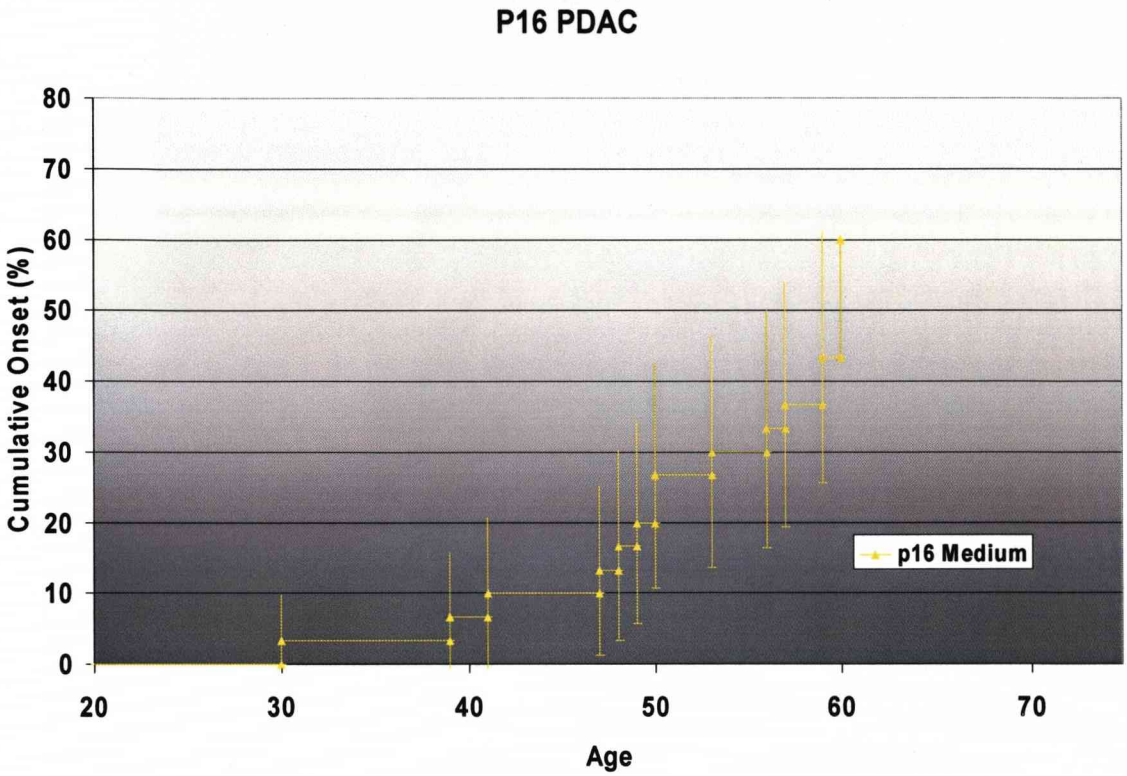


Figure 24: MGMT Methylation vs Age

MGMT methylation is noted to start occurring towards the 40 years old age mark. There results did not, however, achieve stastistical significance (some graphs not shown).

Figure 25: P16 Methylation in PDAC Patient Group



The graph shows the cumulative incidence for p16 methylation increases with age in patients with PDAC.

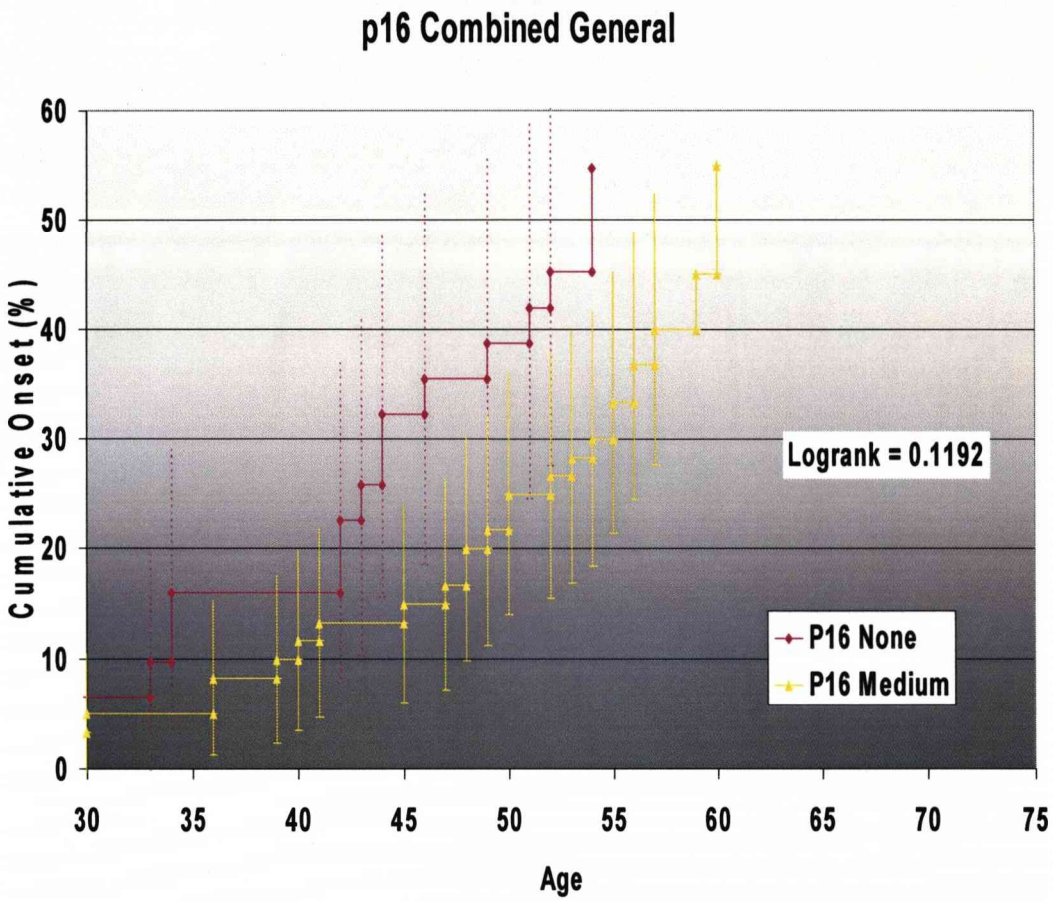


Figure 26: P16 Methylation in Combined General Patient Group

For the methylation combined general analysis, methylation status was merged into either none and medium and all the methylation status for p16 and MGMT are pooled together. Therefore, patients with high methylation status would be allocated into the medium methylation group. In analysing the p16 methylated patients from this group, there is the same trend shown of increasing incidence of methylation with increasing age (Logrank p-value 0.1192).

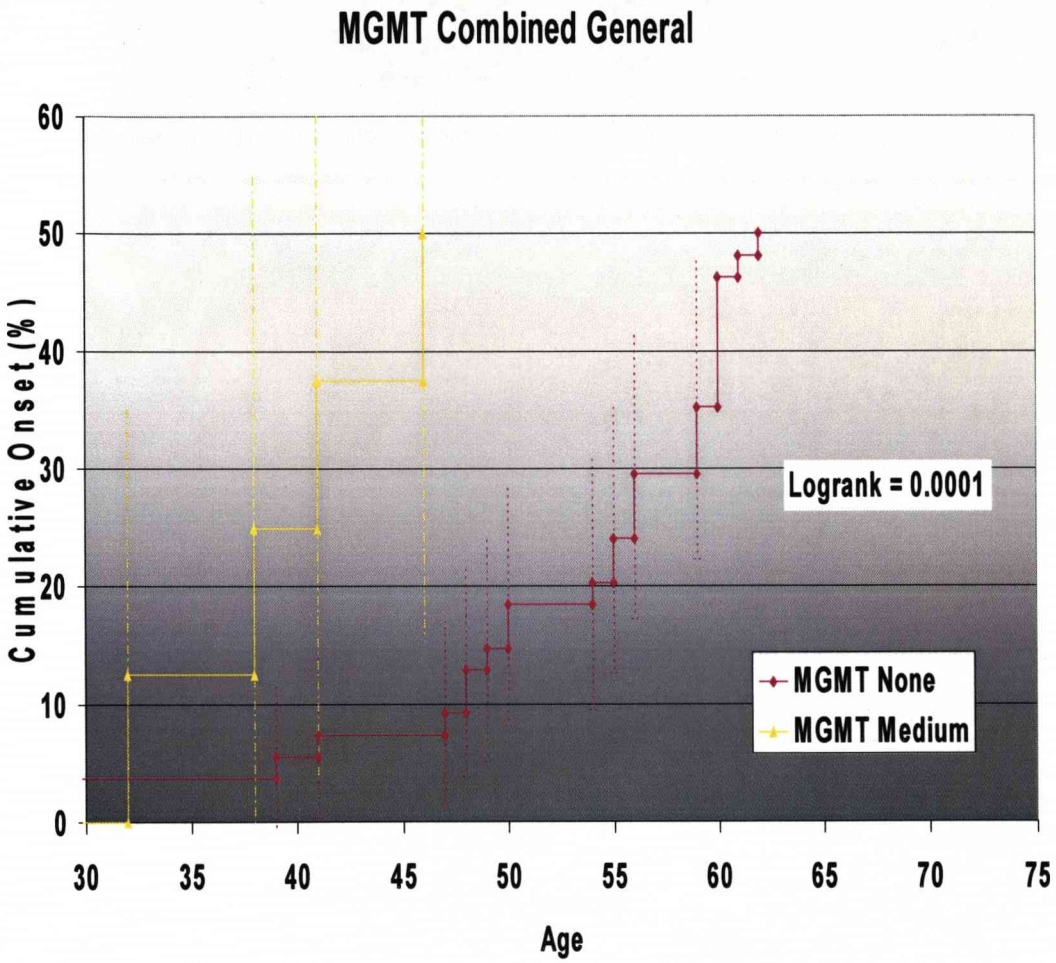


Figure 27: MGMT Methylation in Combined General Patient Group

Cumulative Onset of P16 and MGMT Methylation with Age (Combined General) - More interestingly in the MGMT methylation group, the analysis achieves stastistical significance with a Logrank p-value 0.0001

Discussion

The risk of developing cancer, including pancreatic cancer, increases with the age of a general population. This risk is further exaggerated in patients with hereditary pancreatitis (Howes et al. 2002). It is estimate that ~5-10% of cancers occur with a strong familial association. The association of methylation status and sporadic/ hereditary cancers was looked at by Esteller et al in colon cancer. He reported that in the majority of cases, the p16 and MGMT promoter region methylation were different between sporadic and inherited colon cancers. He postulated that the combination of mismatch repair deficiency and gene silencing by promoter hypermethylation (second hits) can lead to high mutational rates in colon cancer cells (Estellar et al. 2001).

In a study looking into the methylation status of the MGMT gene, Estellar et al found no MGMT promoter methylation in among all normal tissues (including brain, colonic mucosa, breast, pancreas, lung and peripheral blood lymphocytes) tested. Aberrant MGMT hypermethylation was most frequent in primary gliomas and colorectal carcinomas, showing aberrant methylation in ~40% of the cases. A second group of neoplasms with frequent MGMT promoter methylation included lymphomas, non-small cell lung carcinomas, and head and neck carcinomas, where this alteration was found in ~25% of the cases. By contrast, MGMT methylation was found in only 11% (2 of 18) pancreatic carcinomas (Estellar et al. 1999). The authors indicated that the tumour types silencing of the MGMT by aberrant promoter hypermethylation in the study included those with a frequent rate of K-ras mutations (colon, lung, head and neck carcinomas) suggesting the role of loss of MGMT expression leading to an increased susceptibility to

K-ras mutations. However, also found in this study, tumours with known high K-ras mutations (pancreatic carcinomas) were not frequent targets of MGMT promoter hypermethylation, suggesting that inactivation of MGMT is not required for the acquisition of K-ras mutations. This conclusion is in keeping with our finding and the correlation of MGMT hypermethylation with K-ras mutations in all patient groups did not reach stastistical significance.

A follow-up study by Esteller in 2000 reported that a G-A transition mutation of the ras gene showed clear association with MGMT inactivation in colorectal cancer. The study found 71% (36/51) tumours with G-A K-ras mutation had abnormal MGMT methylation compared to 32% (12/37) tumours with other K-ras mutations and 35% (55/156) tumours without K-ras mutations(Estellar et al. 2000). MGMT methylation was also found in equal frequencies in small adenomas <1cm (9/21, 43%), large adenomas >=1 cm (23/44, 53%) and carcinomas (71/174; 40%). This would suggest that hypermethylation of MGMT occurred as an early event in colorectal tumourgenesis. The correlation of the MGMT methylation status with the G-A K-ras mutation indicate that the epigenetic silencing of MGMT by promoter hypermethylation may lead to a particular genetic change in human cancer, specifically G-A transitions in the K-ras oncogene.

Howes et al reported that there is a 4-fold cumulative lifetime risk of developing pancreatic cancer in patients suffering with hereditary pancreatitis (Howes et al. 2002). In a study by House et al, it was suggested that the acquisition of hypermethylation at

tumour suppressor gene promoter sites may contribute to tumour formation and formation within the chronically inflamed gallbladder. Aberrant p16 methylation was found in 30/54 (56%), 2/18 (11%) and 0/15 (0%) gallbladder cancers, chronically inflamed gallbladders and normal gallbladders respectively. MGMT methylation was observed in 7/54 (13%), 3/18 (17%) and 0/15 (0%) gallbladder cancers, chronically inflamed gallbladders and normal gallbladders respectively (House et al. 2003).

We have established in the previous paragraphs that aberrant hypermethylation of promoter CpG islands are an important mechanism for the inactivation of tumour suppressor and repair genes and play a role in tumourgenesis. However, CpG islands are also aberrantly methylated as part of the aging process. Issa et al reported that age related methylation seems to be gene specific, and p16 may be one of the genes not affected. However, this process could be modified by tissue-specific factors (Ahuja et al. 1998). Kang et al studied the frequency of CpG island methylation in several genes including p16 and MGMT, in non-neoplastic gastric mucosa. Two hundred and sixty eight non-neoplastic gastric mucosa samples were analysed and CpG island methylation were found in 10% for p16 and 10.9% for MGMT. This group found that the genes methylated exhibited a general progressive increase as a function of age (DAP-kinase, E—cadherin, p14, THBS1, TIMP-3). Another group of gene methylation did not follow the trend with age (COX-2, GSTP1, MGMT, hMLH1, p16 and RASSF1A) (Kang et al. 2003).

However, there are contradictory reports of p16 methylation related to aging. Waki et al reported the methylation status of tissue samples obtained from autopsies and gastric mucosa (non-neoplastic and neoplastic) obtained from gastric cancer patients undergoing surgery. This group found that methylated p16 was not detected in non-neoplastic cells from tissue samples from autopsies of individuals who were 22 years and younger but was present in 29% (4/14) of individuals who were 45 years or older. These finding suggest p16 methylation in non-neoplastic cells is age-related. P16 methylation was present in 44% (41/94) of non-neoplastic and neoplastic gastric mucosa from patients with gastric cancer (Waki et al. 2002). The methylation of p16 did not correlate with any clinicopathological characteristic.

Issa et al presented finding of two types of CpG islands methylation in colon cancer from patients with ulcerative colitis. Ulcerative colitis is a disease characterized by chronic inflammation and a substantial risk of colon cancer. The two groups were type C, i.e. those that display cancer restricted methylation and type A. i.e. those that are methylated initially in aging normal epithelial (Toyota et al. 1999).

Type A p16 methylation- Issa suggested that p16 gene exon 1 region (known to be hypermethylated in ulcerative colotis patients) behaves as a type A CpG island in the colon, i.e. displays age-related methylation. In his study, he found p16 exon 1 showed substantial levels of methylation in neoplastic and non-neoplastic mucosa of ulcerative colitis patients with high grade dysplasia or cancer. P16 exon 1 methylation in this study averaged 2% in controls, 3 % in ulcerative colitis patients without evidence of dysplasia

(P= 0.6 compared with control), 8% in the normal epithelium of ulcerative colitis patients with high grade dysplasia or cancer (P= 0.007 compared with controls), and 9% in dysplastic epithelium of these same patients (P= 0.03 compared with controls, P= 0.6 compared with nondysplastic epithelium from the same patients) (Issa et al. 2001).

Type C p16 methylation- Issa found the p16 upstream region (critical for transcription) behaves as type C, i.e. methylated in neoplasms only. His study of p16 methylation close to its transcriptional site showed a very low level of methylation (<5%) indistinguishable from background was observed in all patients, with no difference between non-ulcerative colitis controls, ulcerative colitis patients without dysplasia and ulcerative colitis paitents with high grade dysplasia or cancer (Issa et al. 2001).

Issa postulated that disorders characterized by increased cell turnover, such as the chronic inflammation of ulcerative colitis, might be accompanied by higher levels of age-related methylation, that ulcerative colotis could be a disorder of premature aging in the colon. These findings may shed light to the increased risk of pancreatic cancer in patients with hereditary pancreatitis, a disease whereby the patients suffers recurrent attacks of acute pancreatitis resulting to chronic pancreatitis and pancreatic cancer in some patients.

The role of p16 methylation in chronic pancreatitis patients as an indicator of high risk for pancreatic cancer was investigated by Gerdes et al. The group found 10/20 (50%) of chronic pancreatitis specimens haboured PanIN-1a lesions. In 2/10 (20%) of the PanIN-

1a lesions from these specimens, p16 hypermethylation was identified. Hence, p16 alterations, especially promoter methylation, might indicate high-risk precursors in chronic pancreatitis that might progress to cancer (Gerdes et al. 2001).

Chapter 4- Pancreatic Cancer and K-ras

As discussed in the introductory chapter, the early detection of PDAC is a crucial step in the potentially curative process for a patient. Unfortunately, curative treatment is not a frequent option for many patients inflicted by this deadly disease.

Mutations of the K-ras oncogene has been shown to be commonly occurring in PDAC, as discussed and referenced previously. Screenings for PDAC in high-risk populations have been shown to be feasible by Yan et al using 3 novel molecular screening techniques, one of the techniques being the main basis of this thesis. The study showed that the combination of the 3 novel molecular screening techniques increased the discrimination between patients with benign and malignant disease- allowing patients in high risk groups to be stratified from negligible risk to over 50% probability of an early cancer.

Materials

Solutions:

1. TAE (Tris-acetate)- Concentrated stock solution 50x 242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA (pH 8.0)
2. Phosphate buffered solution (GibcoBRL, USA Cat No 14190-094)
3. Ethidium bromide (Sigma, UK Cat No .46047)

Enzymes:

1. AmpliTaq Gold^{TM} (Perkin Elmer, USA Cat No. N808-0241)
2. Bacterial Alkaline Phosphatase (Invitrogen, USA Cat No. 18011-015)

Agarose Gel:

1. Agarose (Sigma, UK Cat. No 13,704-9)

DNA Ladder:

1. 100bp DNA Ladder (Promega, USA Cat No. G2101)

Ready Made Kits:

1. LightCycler- DNA Master SYBR Green 1 (Roche, USA Cat No. 2158817)
2. QIAEX II kit 09/97 (QIAGEN, USA Cat No. 20021)
3. QIAamp^{R}DNA Mini Kit 01/99 (QIAGEN, USA Cat No. 51306)
4. QIAGEN Midi Plasmid Purification Kit (QIAGEN, USA Cat No 12143)
5. pMOSBlue blunt-ended cloning kit (Amersham, USA Cat No. RPN5110)
6. GenElute^{TM} PCR Clean-Up Kit NA (Sigma, UK Cat No. NA1020)
7. LightCycler- Control Kit DNA (Roche, USA Cat No. 2 158 833)
8. LightCycler- DNA Master SYBR Green 1 (Roche, USA Cat No. 2 158 817)

EQUIPMENTS

1. LightCycler, Roche Molecular Biochemicals, UK
2. Sequence detector ABI7700, P.E.Applied Biosystems
3. Polaroid camera, Kodax, UK
4. GeneAmp PCR system 9700, PE Applied Biosystems, USA
5. Centrifuge Spectrafuge 16M, National Labnet Co, USA
6. Pipette, Gilsons, USA
7. Grant hot water bath, Grant Instruments, UK
8. Sorvall Super 2 Centrifuge Sorvall, USA
9. Culton 4300 Innova Incubator Shaker, Bruswick Scientific, USA
10. Culton Incubator Haraeus, Bruswick Scientific, USA

ANALYSIS SOFTWARE

1. Statview, SAS Institute, USA
2. Microsoft Office 2003- Excel, USA

Methods

Recruitment of Families for EUROPAC

Families with HP based in the United Kingdom and Ireland were identified from the EUROPAC Registry. Following informed consent, 10mls of venous blood in EDTA was obtained from all family members over the age of 18 years and whole DNA extracted as per the protocol in the QIAamp^{R} DNA Mini Kit.

Detailed pedigrees were established in conjunction with referring clinicians and pancreatic and clinical genetics specialists from EUROPAC. Family members were questioned to determine whether they have been diagnosed with chronic pancreatitis; their clinical symptoms (abdominal pain, nausea, vomiting, diarrhoea and steatorrhea); together with the number and duration of attacks; age of onset of symptoms and complications of pancreatitis. All the information obtained from the families was corroborated by a questionnaire, completed by the referring clinician, who provided supporting radiological and biochemical investigations of chronic pancreatitis.

Study protocols, questionnaires and consent forms were approved by the Local Research Ethical Committee.

Extraction of DNA from Whole blood, Tissue, Pancreatic Juice and Bile

Clinical samples were collected, after informed consent, from patients undergoing ERCP. The juice samples were spun in a centrifuge at maximum speed room temperature for 15 minutes to collect cells. In samples where cell pellets were seen, these were washed twice with PBS before DNA extraction. In samples where pellets were not visible, whole juices were used for DNA extraction. Diseased tissues were obtained during surgical resection. These samples were initially stored in liquid nitrogen if DNA extraction was not carried out immediately. The DNA was extracted following the protocol in the QIAamp^{R} DNA Mini Kit.

The QIAamp^{R} DNA Mini Kit was used for DNA extraction from the clinical samples- whole blood/ pancreatic juice/ bile/ tissue. For each DNA extraction, initially, 20 µl QIAGEN Protease (or proteinase K) was pipetted into the bottom of a 1.5 ml microcentrifuge tube. Up to 200 µl of whole blood/ pancreatic juice/ bile sample was added to the microcentrifuge tube. If the sample volume was less than 200 µl, the appropriate volume of PBS was added.

200 µl Buffer AL was added to the sample and mixed thoroughly to yield a homogeneous solution by pulse-vortexing for 15 s. The mixture was incubated at 56°C for 10 min. The mixture was briefly centrifuged to remove drops from the inside of the lid. 200 µl ethanol (96–100%) was added to the sample, and mixed again by pulse-vortexing for 15 s. After mixing, the 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid.

The mixture was carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. The cap was closed, and the spin column centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

The QIAamp Mini spin column was carefully open and 500 µl Buffer AW1 was added without wetting the rim. The cap was closed and the spin column centrifuged at 6000 x g (8000 rpm) for 1 min.The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the collection tube containing the filtrate was discarded.

The QIAamp Mini spin column was carefully opened and 500 µl Buffer AW2 added without wetting the rim. The cap was closed and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The QIAamp Mini spin column was placed in a new 2 ml collection tube and the old collection tube with the filtrate was discarded. The spin column was centrifuged at full speed for 1 min.

The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp Mini spin column was carefully opened and 200 µl Buffer AE or distilled water was added. The mixture was incubated at room temperature (15–25°C) for 1 min, and then centrifuged at 6000 x g (8000 rpm) for 1 min. A second elution step with a further 200 µl Buffer AE was performed to increase yields by up to 15%.

ARMS™ Analysis

The detection of K-RAS mutation using ARMS is based on using oligonucleotide primers that are designed to discriminate between target sequences that differ by a single nucleotide. The primers are designed to differ at the 3' terminus nucleotide. This is important because DNA synthesis in the reaction is dependent on correct base pairing at the 3' end. When an ARMS primer is complementary to its target DNA sequence it is extended by a Taq polymerase allowing amplification to proceed thus producing an ARMS product. If the ARMS primer is mismatched at the 3' end, there is no amplification and no product.

Using fluorescently labelled molecular beacons, the ARMS generated PCR products were detected. The molecular beacons consisted of a loop sequence which is complementary to the ARMS amplicon and a stem sequence which is self complementary. At the start of amplification the beacon structure places a fluorophore and quencher molecule in close proximity to each other on the stem and the fluorophore is effectively quenched. As amplification proceeds, ARMS products accumulate and the beacon loop sequence binds to the amplicon. This results in the fluorophore being distanced from the quencher and fluorescence occurs. Signal is detected by the P.E.Applied Biosystems sequence detector (ABI7700). The machine measures the increase in fluorescent signal over time as a function of amplification.

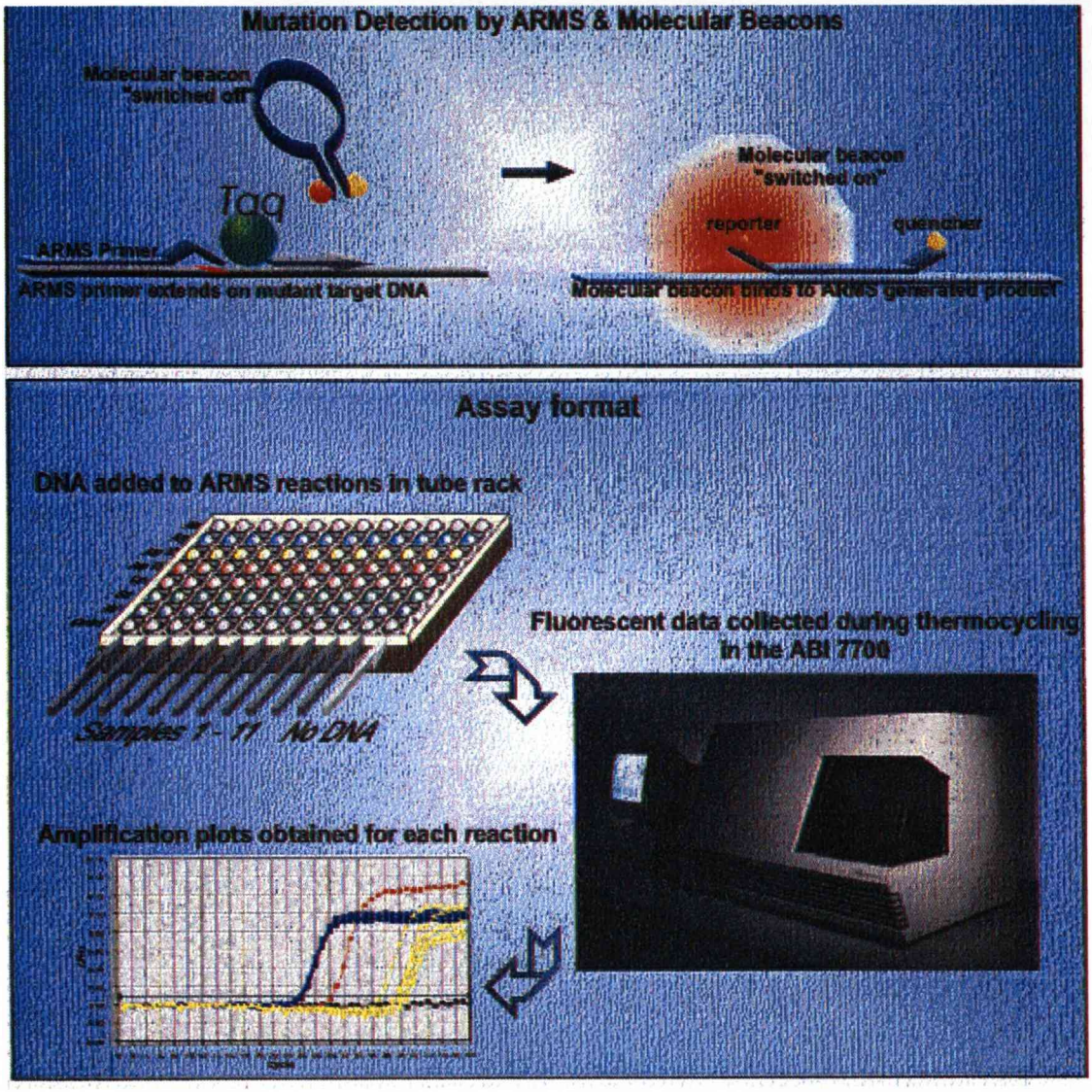


Figure 28: Mutation Detection by ARMS and Molecular Beacons as used at AstraZeneca, Cheshire (Clayton et al. 2000) (taken without permission)

Paired tissue and pancreatic/bile juice DNA samples were analysed for K-RAS mutations using ARMS. DNA samples are added to 8 reactions consisting of 7 K-ras mutant ARMS reactions and a control reaction. The control reaction amplifies a region

of K-ras exon 1 around codon 12 and 13. This controls for yield and degradation by indicating how much K-ras exon 1 is in each sample. A negative (no DNA) control is included in each reaction. The enzyme used for the ARMS reaction was AmpiTaq Gold^{TM} (Perkin Elmer). The beacon used was FAM with the 10x Beacons buffer. A ROX beacon was also used as a passive reference for comparison of the flourescence from the molecular beacons. The enzyme was activated at 94°C for 20 minutes and the reaction was subjected to 50 cycles of 94°C denaturation for 1 minute and 60°C annealing for 1 minute for all the primers listed below.

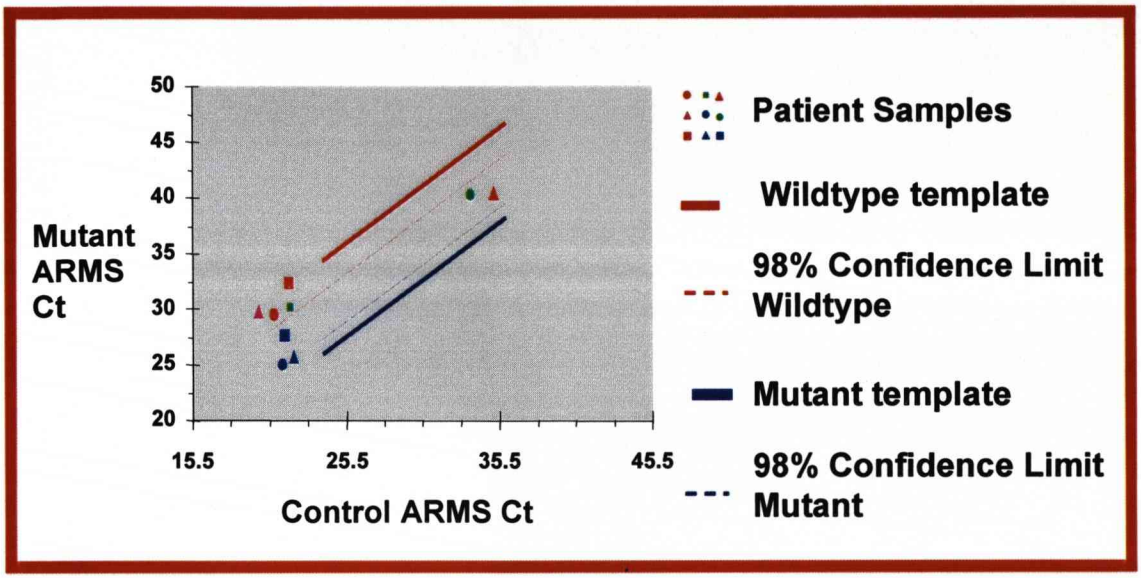


Figure 29: Graph showing K-Ras status after ARMS analysis

PCR primers specific for individual mutations of K-Ras at codon 12 and 13 are used to amplify DNA extracted from pancreatic juice. The number of cycles required to produce detectable product is determined ©. A sequence of K-Ras not carrying the mutation is amplified from the same DNA and the Ct determined (ARMS Control C). The two CT values are plotted against each other and compared to curves produced with varying concentrations of pure wild type (red line) and pure mutant (blue line) K-Ras. If the test sample falls below the 98% confidence limit for wild type, it is classified as mutant.

The primers used were:

Common forward

5' GTA CTG GTG GAG TAT TTG ATA GTG TAT TAA CC 3'

Common reverse

5' CTC ATG AAA ATG GTC AGA GAA ACC TTT ATC 3'

Control (forward)

5' TGA CTG AAT ATA AAC TTG TGG TAG TTG GCG 3'

Cys (forward)

5' CTG AAT ATA AAC TTG TGG TAG TTG GAG CAT 3'

Ser (forward)

5' CTG AAT ATA AAC TTG TGG TAG TTG GAG CCA 3'

Arg (forward)

5' CTG AAT ATA AAC TTG TGG TAG TTG GAG TTC 3'

Val (reverse)

5' TAT CGT CAA GGC ACT CTT GCC TAC GCC TA 3'

Ala (reverse)

5' TAT CGT CAA GGC ACT CTT GCC TAC GCC TG 3'

Asp12 (reverse)

5' TAT CGT CAA GGC ACT CTT GCC TAC GCC TT 3'

Asp13 (reverse)

5' GCT GTA TCG TCA AGG CAC TCT TGC CTA CCT 3'

The results were analysed on the ARMS analysis software (AstraZeneca Diagnostics, Northwich).

Cloning of Mutant K-Ras using pMOSBlue

Prior to setting up the LightCycler™ for K-Ras analysis analysis in our clinical samples, we had to manufacture mutant K-Ras (for Serine, Argenine, Alanine, 13 Aspartate mutations) to use in the initial analysis to test the sensitivity and specificity of our system before proceeding to the analysis of clinical samples.

The pMOSBlue cloning kit was used. This is a blunt-ended cloning kit optimized for the rapid and efficient cloning of all PCR products.

PCR reactions for DNA insert

PCR reactions were carried out using the primer sequences as listed below to produce DNA inserts for cloning.

K-RASFWDCOMMON
5' GTA CTG GTG GAG TAT TTG ATA GTG TAT TAA CC 3'
K-RASREVCOMMON
5'CTC ATG AAA ATG GTC AGA GAA ACC TTT ATC 3'
NEW2F
5' ATA TTA CTG GTG CAG GAC CAT TCT T 3'
NEW 1R
5' CTC CAA CTA CCA CAA GTT TAT ATT 3'
SER-RN
5' CAC TCG AGC CTA CGC CAC TAG CTC CAA CTA C 3'
ARG-RN
5' CAC TCG AGC CTA CGC CAC GAG CTC CAA CTA C 3'
13D (13ASP)-FN
5' GGC TCG AGG AGC TGG TGA CGT AGG CAA GAG T 3'
ALA-FN
5' GGC TCG AGG AGC TGC TGG CGT AGG CAA GAG T 3'

The enzyme used for this PCR was AmpliTaq Gold^{TM} with 10x PCR Gold Buffer and $MgCl_2$ solution.

The mastermix components for each 50 µl reaction are listed below:

10x PCR buffer	1µl
2.5mM dNTP	1µl
Primers (1pmol/ul)	2µl
Taq	0.2µl
$MgCl_2$	12µl
Water	27.8µl
Template DNA	2µl

The enzyme was activated at 94°C for 12 minutes, and then the reaction was subjected to 35 cycles of amplification at 94°C for 30 seconds, followed by annealing at 65°C for 30 seconds. This was followed by chain elongation at 72°C for 1 minute. The reaction was kept at 72°C after the 35 cycles for a further 7 minutes before being cooled to 4°C.

The PCR products (5µl) was loaded onto a 2% agarose gel stained with ethidium bromide (1µl) and ran in 1x TAE and visualised under UV illumination.

The sizes of the PCR products are as follows:

NEW1R	127bp
NEW2F	62bp
ALA	150bp
ARG	150bp
SER	150bp
13ASP	150bp

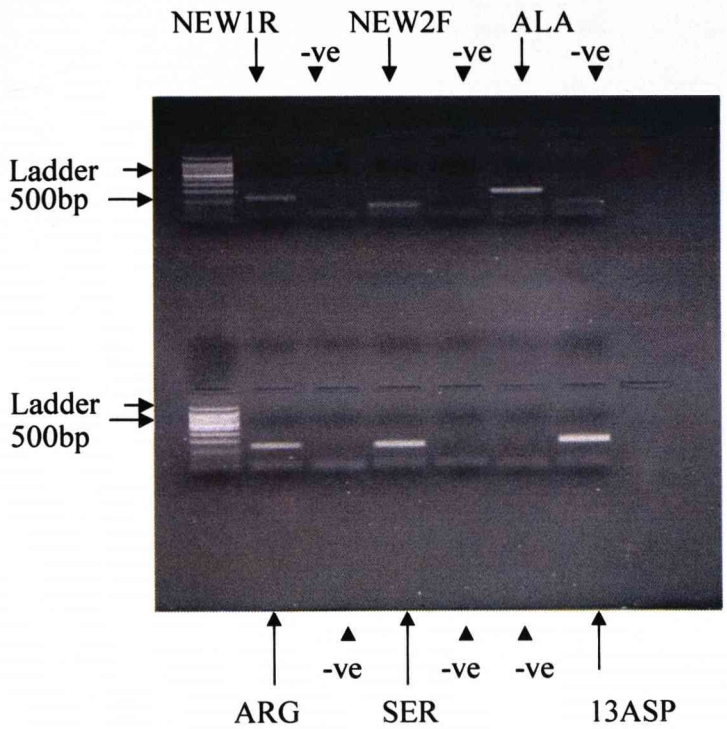


Figure 30: Agarose gel of PCR DNA inserts for Cloning

The gel above shows the PCR products following amplification with the named primers (as indicated) in preparation for cloning.

The PCR inserts were purified with the Qiaex 11 kit for optimal cloning efficiencies.

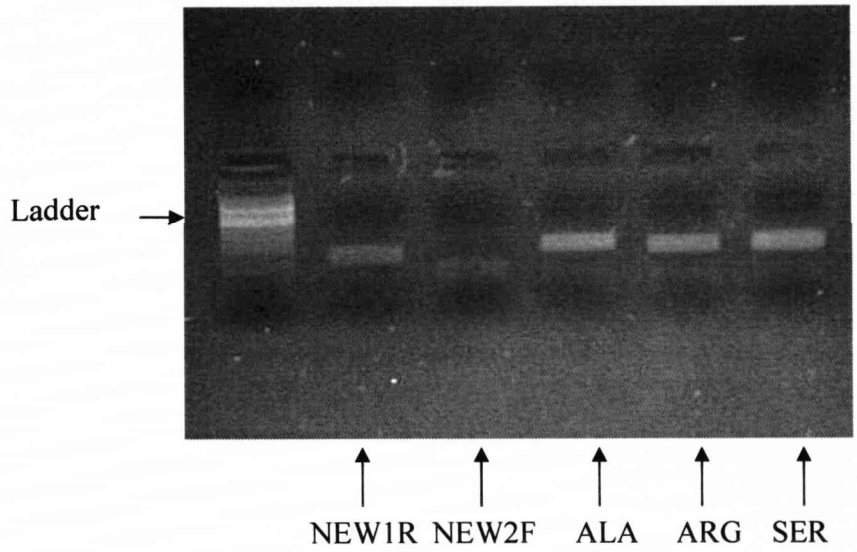


Figure 31: Agarose gel of PCR inserts after QIAEXII purification

The agarose gel shows that the PCR products have not been lost following purification in preparation for cloning.

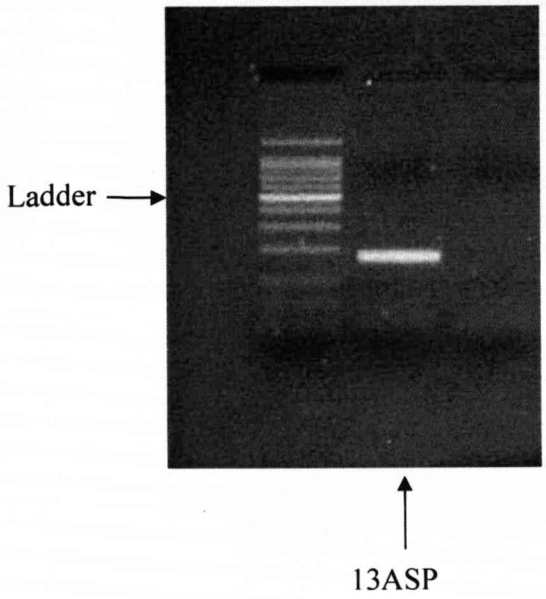


Figure 32: Agarose gel of PCR inserts after QIAEXII purification

The agarose gel shows that the PCR products have not been lost following purification in preparation for cloning.

pK Reactions

The amount of PCR inserts required for the pK reaction was calculated using the formula

$\frac{Z \times 50}{2887} \times \frac{2.5}{1}$ = ___ ng insert (where Z = size of insert in bp)

The ratio recommended of vector: insert molar ratio by the manufacturer ranges from 1:1 to 1:2.5.

The 10 µl pK reaction mix is set up as follows in 1.5 ml Eppendorf tubes:

Reaction mix for NEW1R, NEW2F and Control inserts:

10x pK buffer	1µl
100mM DTT	0.5µl
pK enzyme mix	1µl
Insert	2µl
Water	5.5µl

(NEW1R for Colony 1 and NEW2F for Colony 2)

Reaction mix for ALA, ARG, SER, 13ASP inserts:

10x pK buffer	1µl
100mM DTT	0.5µl
pK enzyme mix	1µl
Insert	1µl
Water	6.5µl

(ALA for Colony 3, ARG fro Colony 4, SER for Colony 5 and 13ASP for Colony 6)

The reactions were incubated at 22°C for 40 minutes and centrifuged briefly to collect the contents at the bottom of the tubes. To heat inactivate the kinase enzyme, the reactions were incubated at 75°C for 10 minutes, cooled on ice for 2 minutes (to avoid inactivation of the ligase in the ligation reaction) and finally, centrifuged briefly again to collect the condensate.

Ligation

The vectors (50 ng) were ligated with the pK reactions and the ligation reactions (12 µl) were prepared as follows:

pK reaction mixture	10µl
pMOSBlue vector (50ng/µl)	1µl
T4 DNA ligase (4 Weiss units)	1µl

The ligation mixtures were incubated at 22 °C overnight.

Transformation

The pMOSBlue competent cells were thawed and 20µl were pipetted into the required number of pre-chilled microfuge tubes on ice. 1µl of ligation mix was added directly to each microfuge tube and the tubes were left on ice for 30 minutes. The cells were then placed in a water bath at 42°C for exactly 40 seconds and returned to ice for a further 2 minutes. 80µl of room temperature SOC medium was added to each tube and the tubes were shaken at 200-250rpm at 37 °C for 1 hour.

X-gal (35µl of 50mg/ml) and IPTG (20µl 100mM) were spreaded on the L agar antibiotic plates and left to soak for 30 minutes prior to plating. 50µl of each transformation was spreaded on to the L agar plates (*see below). The plates were incubated inverted at 37 °C.

*(The L agar antibiotic plates were prepared using 10g tryptone, 5g yeast extract, 10g sodium chloride, 15g agar in 1 litre of solution. The solution was sterilized by autoclaving Ampicillin was added to a final concentration of 50µg/ml and tetracycline to 15µg/ml. Fresh filtered sterilized stock solutions were added after the medium had cooled to <50 °C.)

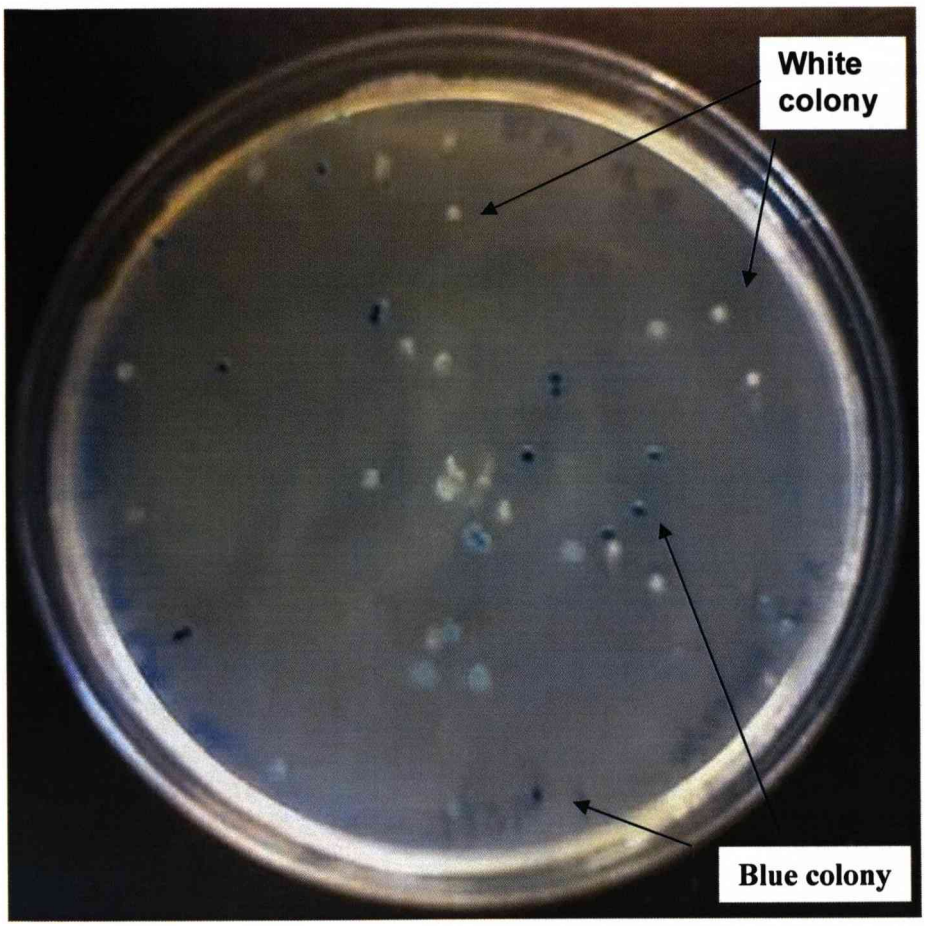


Figure 33: Picture of blue-white colonies from pMOSBlue cloning kit

An example of blue-white colonies following successful integration of mutant K-ras DNA.

Colony Harvesting and PCR Screening

The white colonies were harvested and

i) Replated or

ii) Mixed in 20µl of water, heated to 95°C for 10 minutes, spun and the supernatant collected ready for the PCR screen.

Colony PCR Screening

The enzyme used for this PCR was AmpliTaq Gold^{TM} with 10x PCR Gold Buffer and $MgCl_2$ solution.

The mastermix components for each 30 µl reaction are listed below:

For NEW1R, ARG, SER screen

dNTP	1µl
T7 promoter	1µl
KRASFWDCOMMON primer	1µl
$MgCl_2$	4µl
10x buffer	2.5µl
Water	10.4µl
Enzyme	0.1µl
PCR product	5µl

For NEW2F, ALA, 13D screen

dNTP	1µl
T7 promoter	1µl
KRASREVCOMMON primer	1µl
$MgCl_2$	4µl
10x buffer	2.5µl
Water	10.4µl
Enzyme	0.1µl
PCR product	5µl

The enzyme was activated at 94°C for 12 minutes, and then the reaction was subjected to 35 cycles of amplification at 94°C for 30 seconds, followed by annealing at 55°C for 30 seconds. This was followed by chain elongation at 72°C for 1 minute. The reaction was kept at 72°C after the 35 cycles for a further 7 minutes before being cooled to 4°C. The PCR products were loaded on an ethidium bromide stained 2% agarose gel.

The PCR products are of the following sizes:

NEW1R	126bp
NEW2F	62bp
ALA	174bp
ARG	147bp
SER	147bp
13ASP	174bp

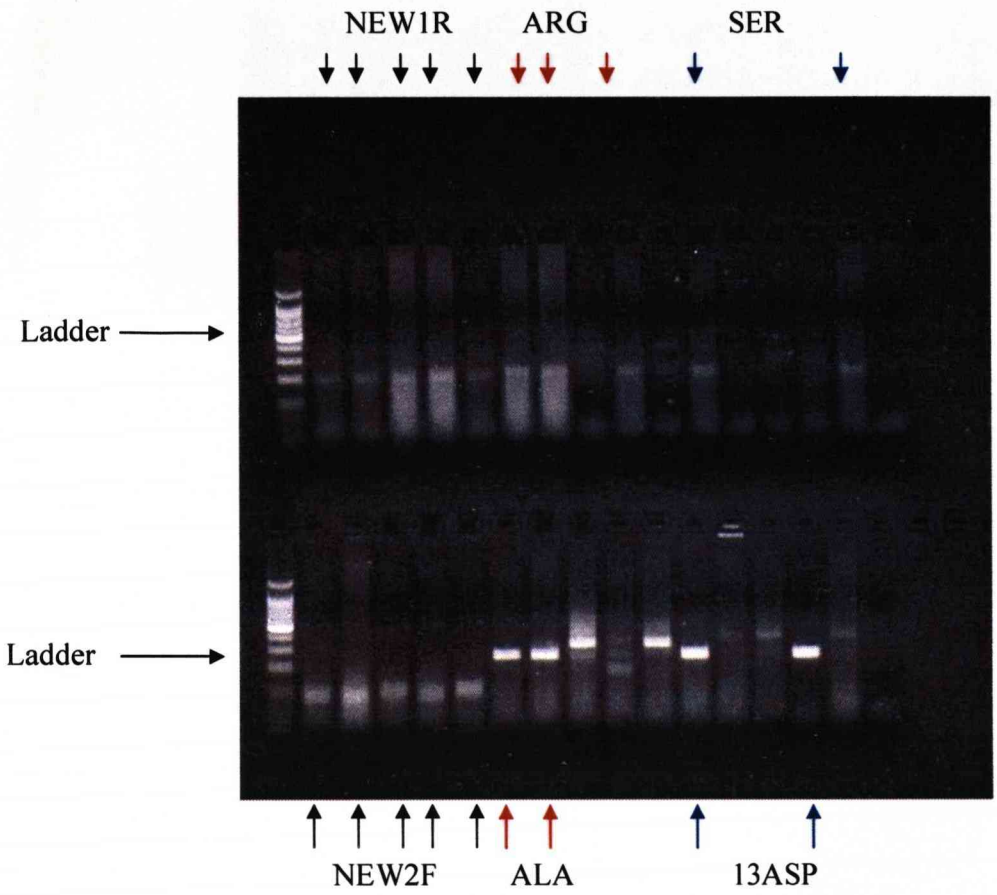


Figure 34: Agarose gel of PCR Screen for mutant Kras constructs of the correct orientation.

Following cloning, DNA was extracted from the colonies and screened for correct orientation. The agarose gel above shows (as indicated in the labelled lanes) colonies with the orientation for ALA, 13ASP, ARG and SER colonies.

The colonies with the correct constructs were purified using the Qiagen Midi Plasmid Purification Kit following the manufacturer's protocol. The purified products were loaded on an ethidium bromide stained 2% agarose gel for concentration estimation.

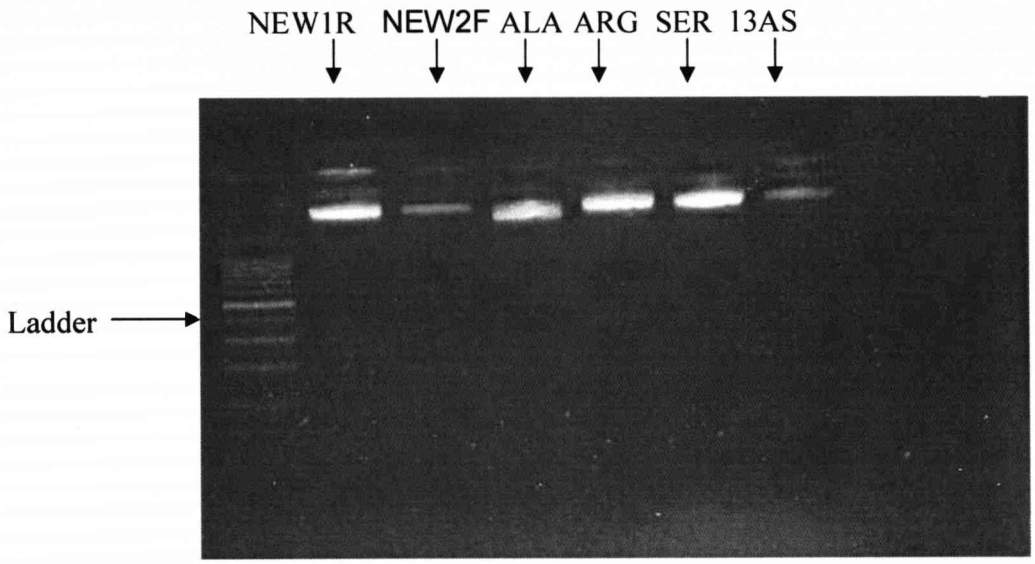


Figure 35: Agarose gel of PCR with mutant Kras constructs of the correct orientation after plasmid purification

The agarose gel shows the DNA with the correct construct orientation still present after purification.

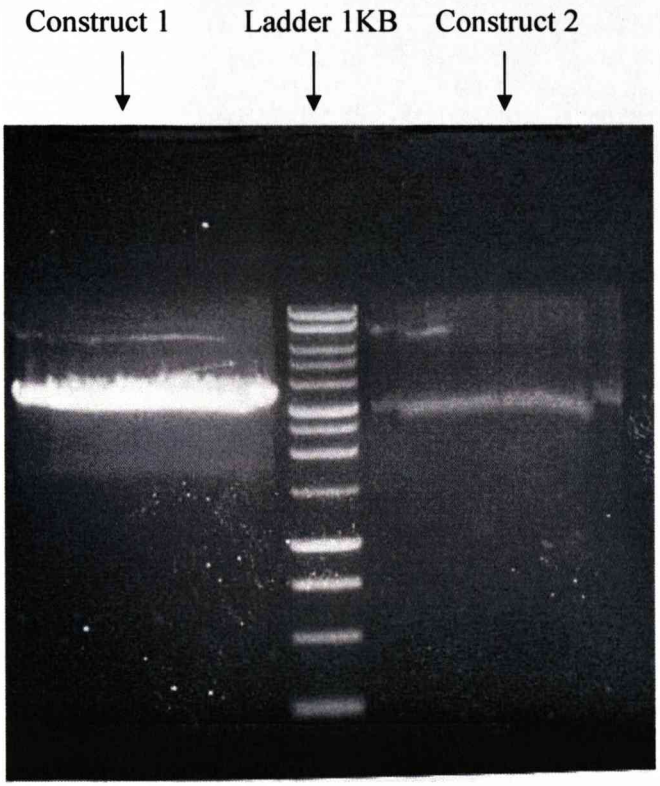


Figure 36: Agarose gel showing digestion of Consrtuct 1 (NEW1R) and 2 (NEW2F) with SalI and PSTI enzymes

Construct products from Colony 1 (NEW1R) and 2 (NEW2F) were double digested with SalI and PSTI enzymes. The digestion mixtures were incubated at 65°C for 1 hour. The digested products were loaded on an ethidium bromide stained 0.8% agarose gel with a 1Kb ladder.

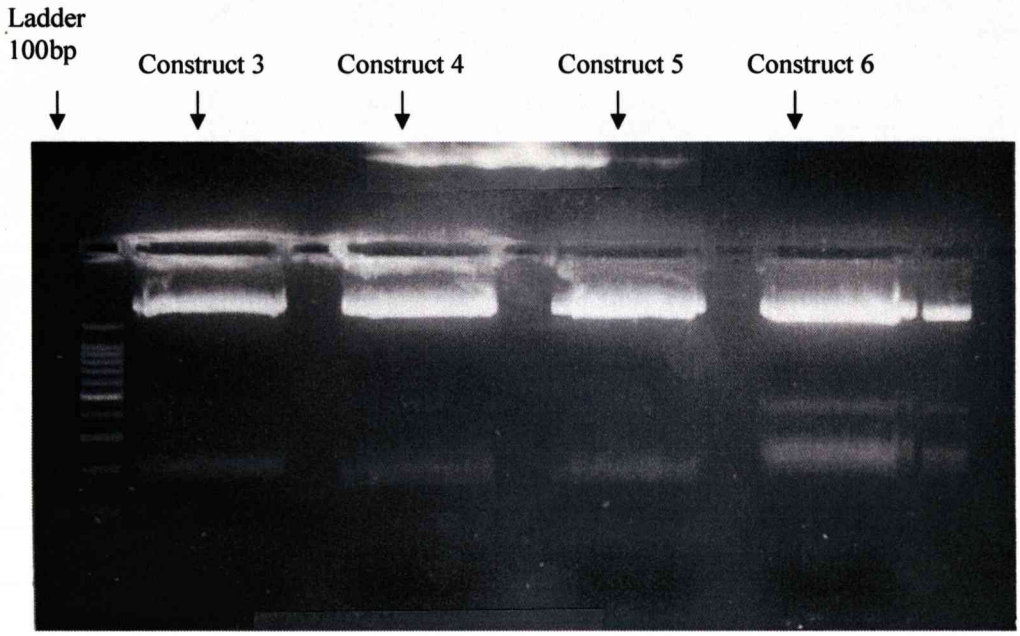


Figure 37: Agarose gel showing digestion of Construct 3 (ALA), 4 (ARG), 5 (SER) and 6 (13ASP) with XhoI and PSTI enzymes

Construct products from Colony 3 (ALA), 4 (ARG), 5 (SER) and 6 (13ASP) were double digested with XhoI and PSTI enzymes. The digestion mixtures were incubated at 65°C for 1 hour. The digested products were loaded on an ethidium bromide stained 2% agarose gel with a 100bp ladder.

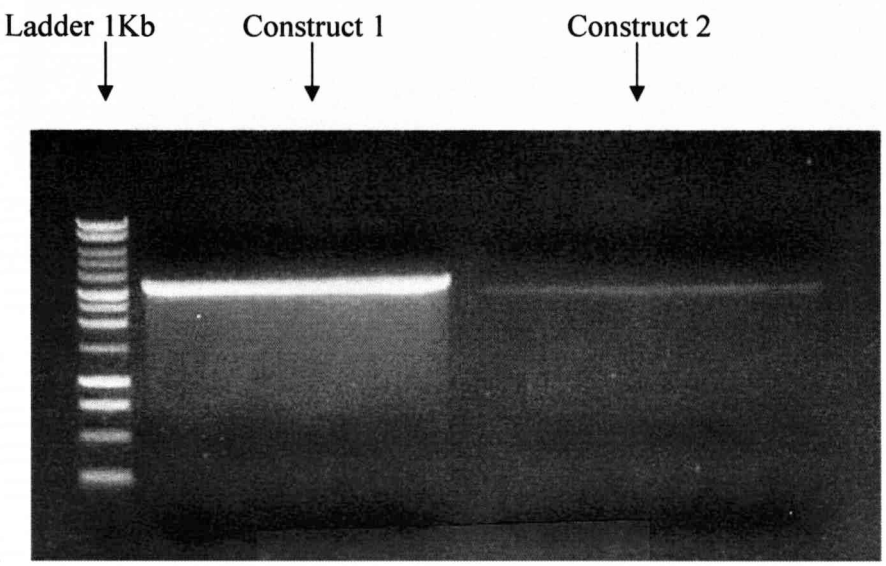


Figure 38: Agarose gel showing Constructs NEW1R and NEW2F after dephosphorylation

Constructs from Colony 1 (NEW1R) and 2 (NEW2F) were dephosphorylated with bacterial alkaline phosphatase following manufacturer's instructions at 65°c for 1 hour. Some of the dephosphorylated products were loaded onto a 0.8% agarose gel with a 1Kb ladder.

The constructs were religated in the following combinations following the pMOSBlue manufacturer's protocol as previously described.

Construct 1 (NEW1R) and Construct 3 (ALA)
Construct 1 (NEW1R) and Construct 6 (13ASP)
Construct 2 (NEW2F) and Construct 4 (ARG)
Construct 2 (NEW2F) and Construct 5 (SER)

The colonies with the mutant Kras constructs were screen by PCR for the correct orientation. The primers used PCR mastermix and the agarose gels are shown below.

Primers used for mutant Kras screening:

5'-GTA CTG GTG GAG TAT TTG ATA GTG TAT TAA CC-3' F Common
5'-CTC ATG AAA ATG GTC AGA GAA ACC TTT ATC-3' R Common
5' CAC TCG AGC CTA CGC CAC TAG CTC CAA CTA C 3' SER-RN
5' CAC TCG AGC CTA CGC CAC GAG CTC CAA CTA C 3' ARG-RN
5' GGC TCG AGG AGC TGG TGA CGT AGG CAA GAG T 3'13D (13ASP)-FN
5' GGC TCG AGG AGC TGC TGG CGT AGG CAA GAG T 3' ALA-FN

The R (Reverse) common primer was used with FN (Forward) primers and the F common primer was used with the RN mutation specific primers.

dNTP	1µl
Primer	2µl
$MgCl_2$	4µl
10x Gold buffer	2.5µl
AmpliTaqGold	0.1µl
Water	10.4µl
DNA/mutant sequence	5µl

The enzyme was activated at 94°C for 12 minutes, and then the reaction was subjected to 35 cycles of amplification at 94°C for 30 seconds, followed by annealing at 55°C for 30 seconds. This was followed by chain elongation at 72°C for 1 minute. The reaction was kept at 72°C after the 35 cycles for a further 7 minutes before being cooled to 4°C. The PCR products were loaded on an ethidium bromide stained 2% agarose gel.

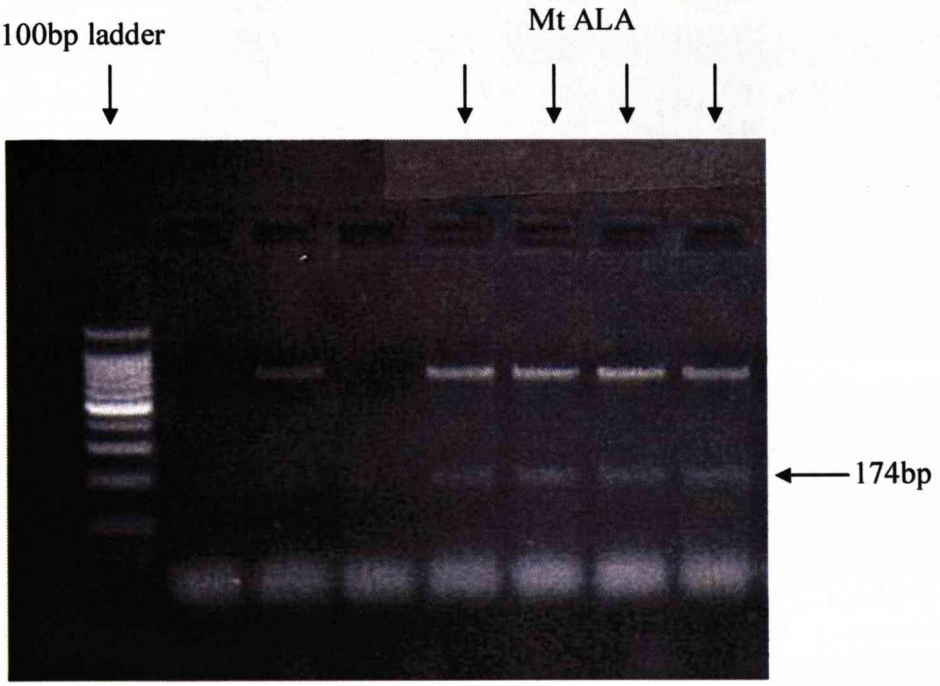


Figure 39: Agarose gel of Kras with mutant ALA

The agarose gel shows successful production of DNA with mutant K-ras alanine sequence.

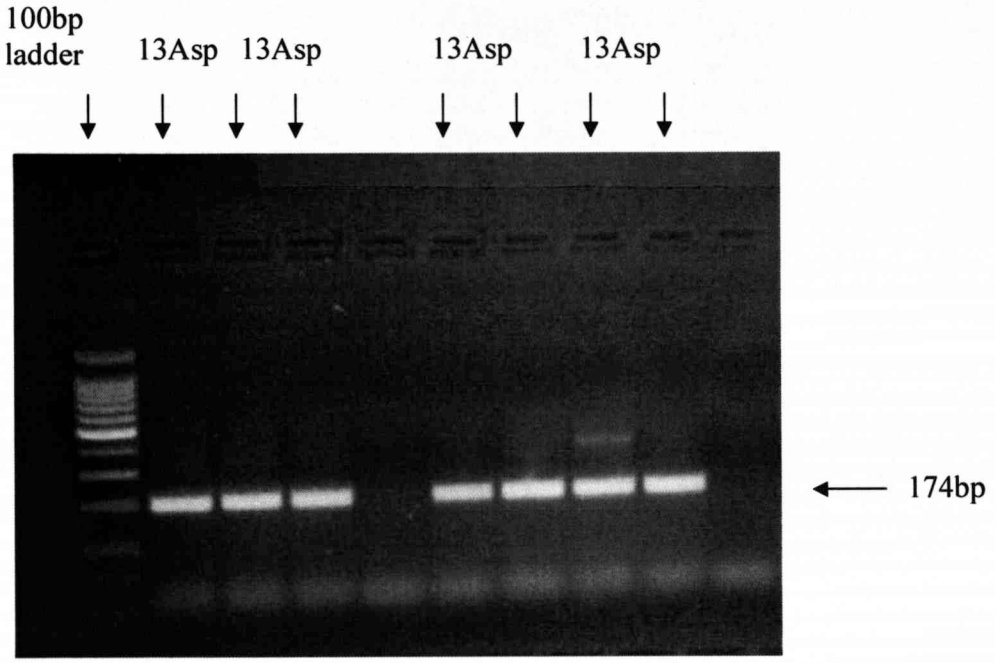


Figure 40: Agarose gel of Kras with mutant 13Asp

The agarose gel shows successful production of DNA with mutant K-ras 13 Aspartate sequence.

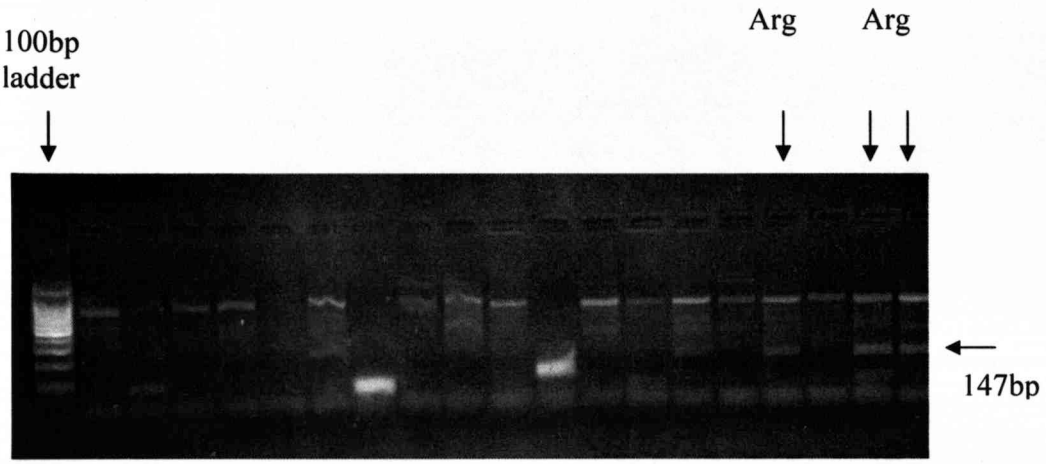


Figure 41: Agarose gel of Kras with mutant Arg

The agarose gel shows successful production of DNA with mutant K-ras arganine sequence.

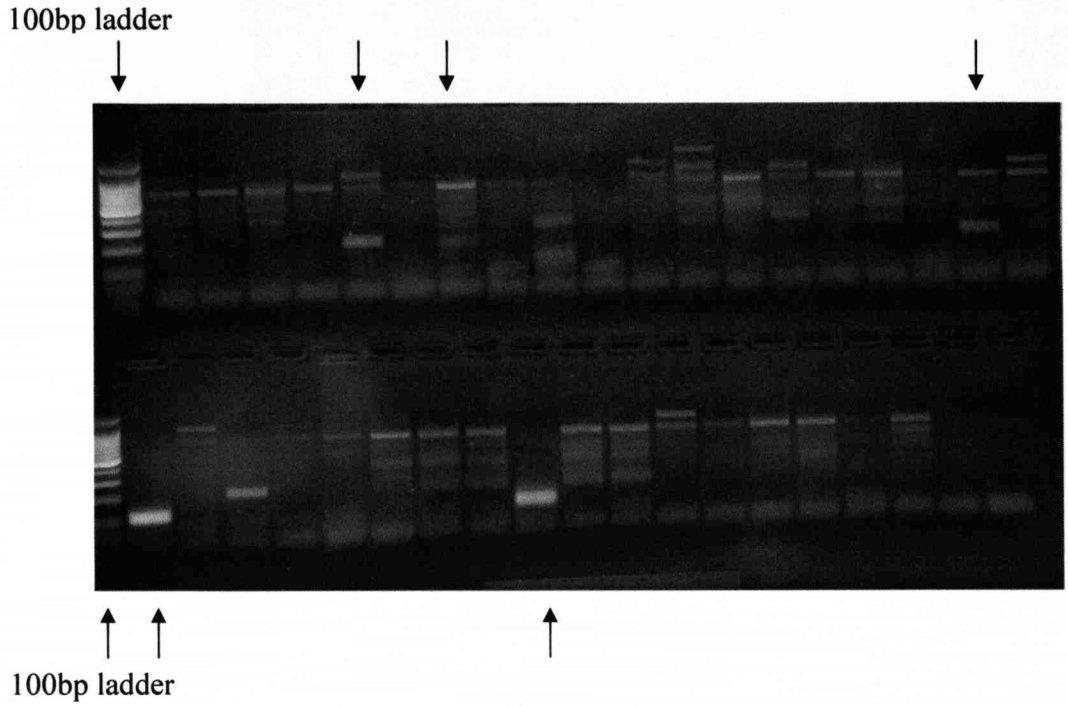


Figure 42: Agarose gel of Kras with mutant Ser (147bp)

The agarose gel shows successful production of DNA with mutant K-ras serine sequence.

The mutant Kras sequences are ready for use in the calibration and quantification process in setting up the LightCycler™ machine.

Setup of LightCycler for K-Ras Analysis and K-Ras Analysis

The LightCycler Instrument

The Lightcycler Instrument of Roche Molecular Biochemicals is a thermocycler for the rapid analysis of PCR applications. This innovative machine allows for carrying out and simultaneously evaluating PCR experiments.30 amplification steps including anaylsis of results can be carried out in less than 30 minutes.

Fluorimetric analysis of the PCR products formed is performed as real-time measurement either continuously or at a specifically defined time during each PCR cycle. The analysis can be monitored online by the LightCycler's software, directly during the reaction.

Components of the LightCycler

LightCycler Instrument
Sample Carousel (for 1.5mm capillary) premounted in LightCycler Instrument
LightCycler Capillaries (96 capillaries and stoppers/box)
32 LightCycler Centrifuge Adapters in an aluminium cooling block

Figure 43: Picture of the LightCycler from Roche Molecular Biochemicals (taken without permission from LightCycler Operator's Maunal Version 3.0 May 1999)

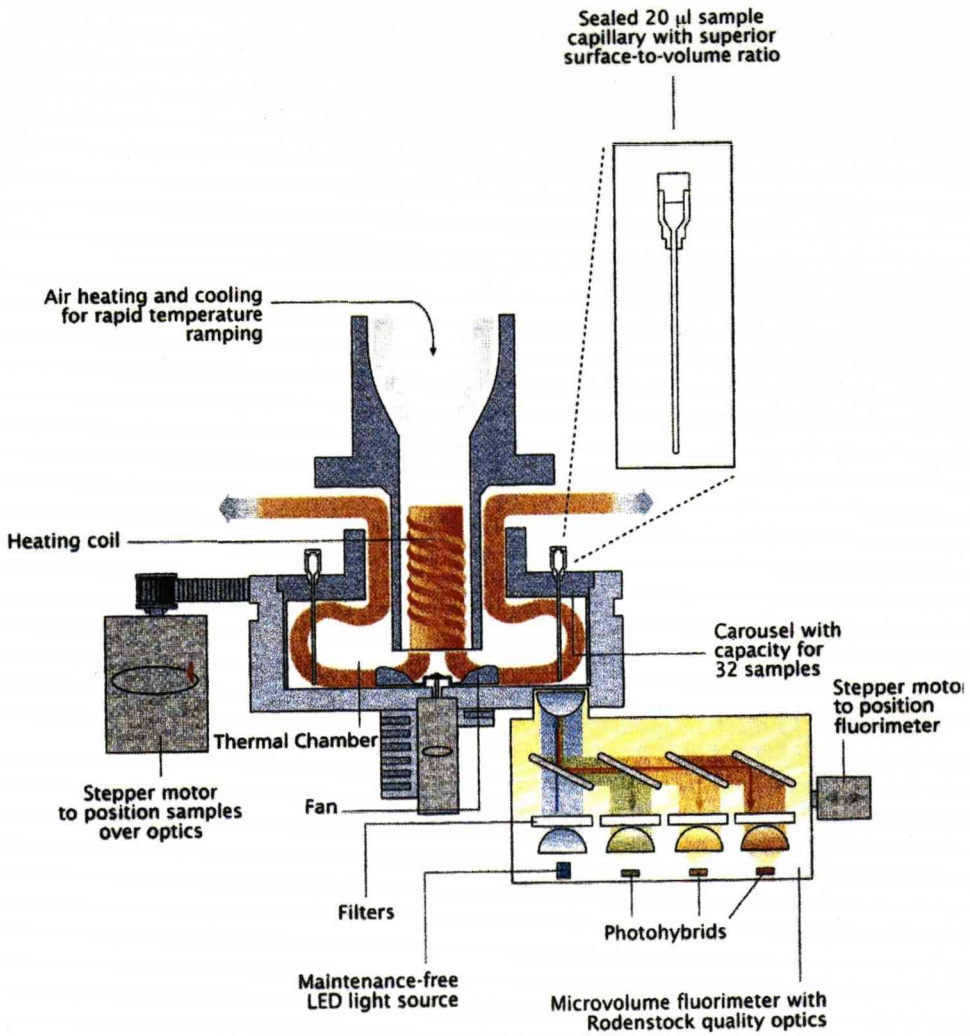


Figure 44: Schematic diagram of the LightCycler (taken without permission from LightCycler Operator's Manual Version 3.0 May 1999)

*The LightCycler consists of an upper unit and a lower unit. The upper unit contains the heating coil. The lower unit contains the thermal chamber, fluorimeter, drive units, electronic boards and power supply. The various elements are mounted on a 10-mm cast aluminium base plate. This guarantees stability, especially for the thermal chamber and flourimeter.

Hot or ambient temperature air, introduced into the thermal chamber, regulates the temperature of the sample capillaries. A heating coil heats the air, which is them fed into the chamber by the fan. The fan ensures efficient air circulation and temperature homogeneity during the heating cycle.

During the cooling cycle, the fan operates at a higher speed to ensure adequate cooling. During measurements, a stepper motor rotates the sample carousel to position the capillary tip precisely at the focal point of the fluorimeter optics. The fluorimeter itself is positioned radially to the maximum signal to compensate for any radial deviation of the capillary tip.

For online display, data are transmitted to and from the PC via a serial interface.

Temperature is controlled with hot air and air at ambient temperature. Arying the voltage supplied to the heating coil regulated the temperature. A sensor provides reference valus for control purposes.

During the heating phase, the fan in the thermal chamber operates at low speeds to ensure homogenous distribution of temperature. During the cooling phase, the fan operates at higher speeds so that the capillaries and the heating coil can be cooled efficiently.

The two sensors are integrated into the LightCycler to prevent unduly high temperatures:

- Sensor I is located in the themal chamber and switches off the heat when a temperature of 125°C has been reached.
- Sensor II monitors the temperature on the aluminium base plate, and switches off the entire LightCycler to protect the electronics when the temperature exceeds 55°C.

A three-channel fluorimeter is used for detection purposes. A blue diode (LED) with maximum emission of 470nm serves as the energy source for sample excitation. Screens are used to diffuse the light emitted by the LED to ensure uniformity. The exhaust and ventilation channels have been designed to prevent ambient light from entering directly into the thermal chamber. Flourescence is detected at 530nm, 640nm and 710nm with the aid of photohybrids.

The thermal chamber and the fluorimeter are palced optimally for sample measurement. Only the sample carousel, which holds the capillary, rotates during measurement of the various samples.*

(** extracted from LightCycler Operators Manual Version 3.0 Roche Molecular Biochemicals, without permission)

The LightCycler Software

The LightCycler was supplied with pre-installed software to allow for the analysis of samples. When programming a Run for an experiment, a Programming Screen appears. The Programming Screen allows for:

- Defining the parameters of a PCR protocol
- Starting a PCR run or
- Viewing online a PCR experiment that is currently in progress.

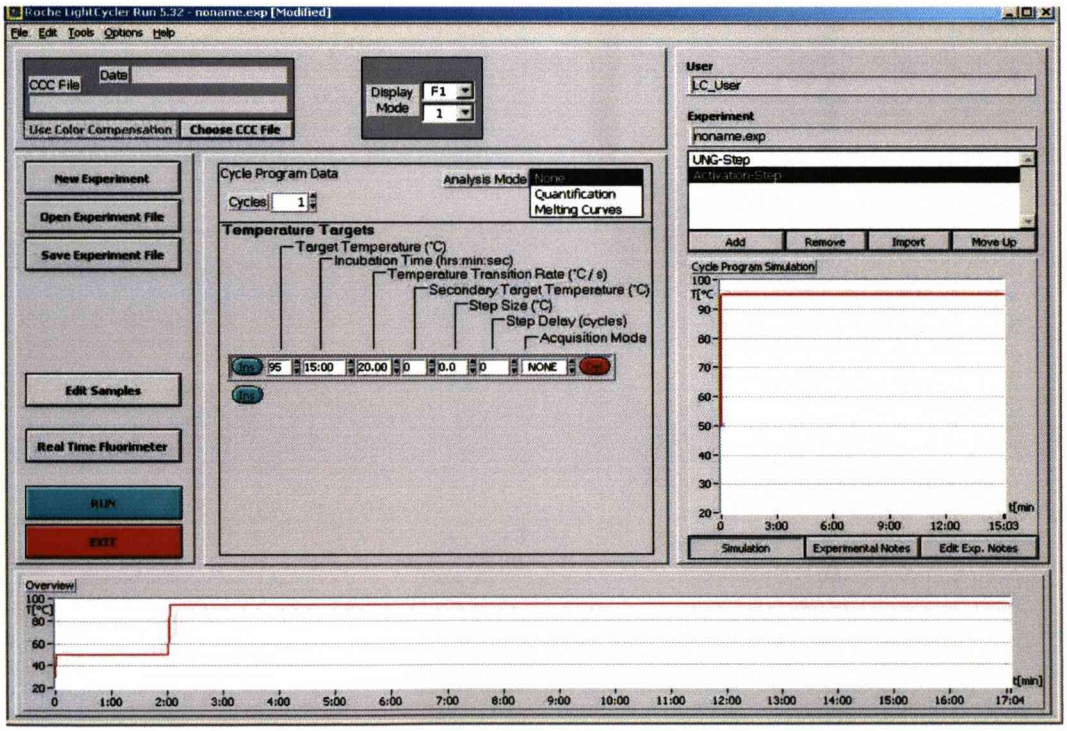


Figure 45: Diagram of the Programming Screen

An Experimental Protocol contains one or more Cycle Programs. A typical Experimental Protocol contains four programs:

- Program 1: Initial Denaturation
- Program 2: Amplification
- Program 3: Melt and
- Program 4: Cooling

A Program (e.g. Cycle Program) contains seceral Temperature Segments, each of which defines the time and parameters that will be used for denaturation, annealing, extension and/or melting, cooling as well as fluorescence acquisition mode used to monitor the amplification signal.

Data Analysis

The LightCycler Data Analysis (LCDA) sortware allows a LightCycler used to analyze quantification data or melting curve data acquired during a LightCycler reaction. The LCDA software can display and analyze in two formats:

- **Quantification**: Estimates the original number of target DNA copies in a sample by comparing it to at least two known concentration of a standard. It displays the amplification profile of a PCR.
- **Melting Curve**: Displays a fluorescence curve profile obtained during a slow denaturation of PCR products, and includes options for differentiationg melting curves to give melting peaks, integrating the area under the melting peaks, and defining the melting temperature of amplified products.

Set-up for Kras Analysis

Before utilizing the LightCycler for formal analysis of patient clinical samples for the presence/ absence of Kras mutations, we optimized the PRC reactions for the Kras primers using the LightCycler. The primers used for the Kras reactions are as follows:

5' CTG AAT ATA AAC TTG TGG TAG TTG GAG TTC 3'	F G12R
5' CTG AAT ATA AAC TTG TGG TAG TTG GAG CCA 3'	F G12S
5' CTG AAT ATA AAC TTG TGG TAG TTG GAG TAT 3'	F G12C
5' TAT CGT CAA GGC ACT CTT GCC TAC GCC TAC GCC TT 3'	R G12D
5' TAT CGT CAA GGC ACT CTT GCC TAC GCC TAC GCC TA 3'	R G12V
5' TAT CGT CAA GGC ACT CTT GCC TAC GCC TAC GCC TG 3'	R G12A
5' GCT GTA TCG TCA AGG CAC TCT TGC CTA CCT 3'	R 13ASP
5' GTA CTG GTG GAG TAT TTG ATA GTG TAT TAA CC 3'	F Common
5' CTC ATG AAA ATG GTC AGA GAA ACC TTT ATC 3'	R Common
5' TGA GTG AAT ATA AAC TTG TGG TAG TTG GCG 3'	F Control
5' CTG TAT CGT CAA GGC ACT CT 3'	R Control

Primers specific for each mutation were used in combination with the appropriate common primer. The Reverse (R) common primer was used with Forward (F) mutation specific primers and the F common primer was used with the R mutation specific primers. The control primers amplify both mutant and wild type sequences.

The PCR reactions were set up using the LightCycler ready made kits listed previously and the reactions conditions are as follows:

95°C	10 minutes
95°C	2 seconds*
61°C	20 seconds*
72°C	20 seconds*
81 °C	10 seconds*
*60 cycles	

Melting curve analysis was performed from 72 to 95 °C to verify that pure PCR products were produced.

The melting curves for the Kras mutants are listed below:

12Asp	~81 °C
12Cys	~82 °C
12Ala	~81 °C
12Arg	~84 °C
12Val	~82 °C
12Ser	~83 °C
13Asp	~82 °C
Control	~78-82 °C

The melting temperature of the products from the LightCycler reactions are checked to ensure that there is no contamination and that the correct products are being analysed.

Following optimization of the LightCycler for the primers, the PCR sensitivity for the detection of mutant Kras sequences was then analyzed. Mutant Kras sequences from the cloned Kras or from know cell lines with known Kras mutation were multiply diluted and analysed with the LightCycler to confirm detection sensitivity to a dilution of 1: 10000. Again, pure PCR products were confirmed from the melting point analysis.

Samples used for analysis of mutant Kras sequences:

12Asp	Panc-1 cell line
13Asp	Cloned
12Ser	Cloned
12Val	CFPac cell line
12Arg	Cloned
12Cys	MiaPACA cell line
12Ala	Cloned

Statistical analyses of these dilutions were performed and graphs were produced for the individual Kras mutations that would enable future anaylses to be plotted on them. This would allow confirmation of the clinical samples analyzed for Kras status- wild type or mutant.

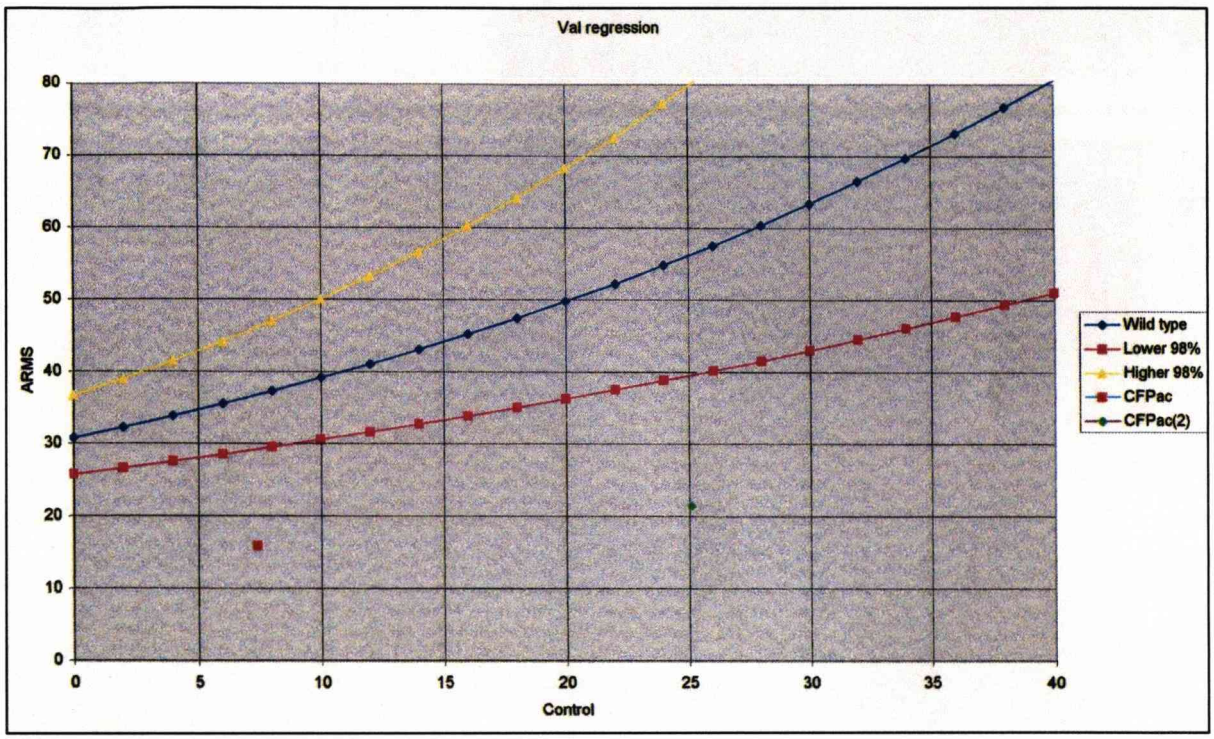


Figure 46: Graph to determine the Kras Valine mutation status. Markers on graph indicate mutant Valine samples

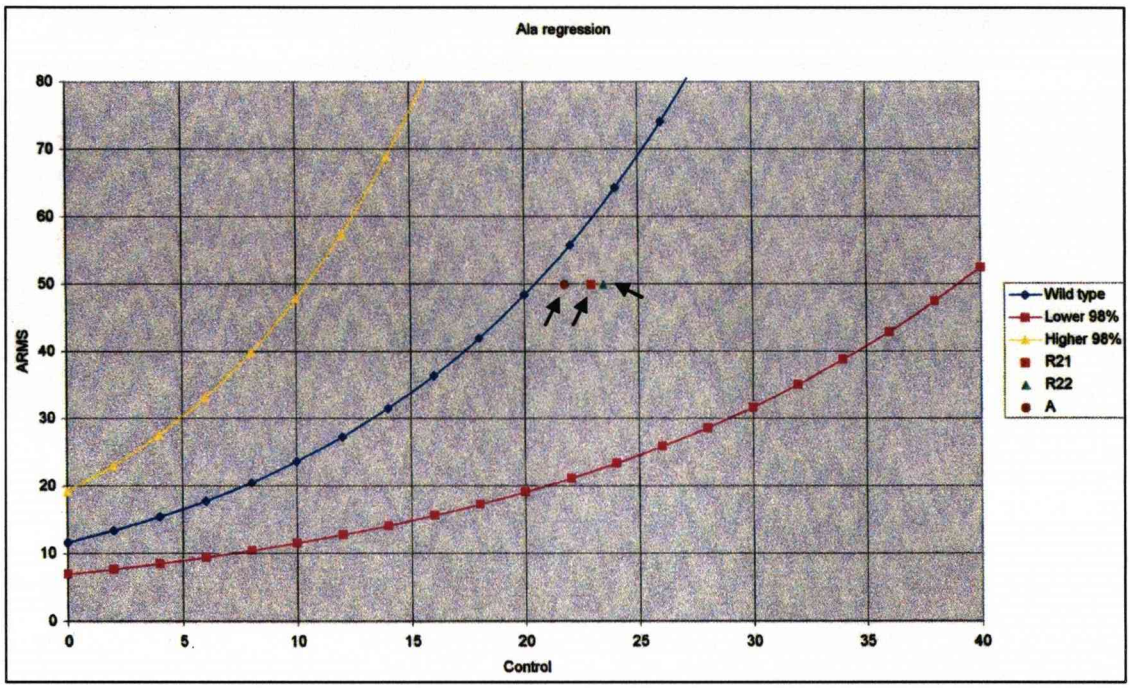


Figure 47: Graph to determine the Kras Alanine mutation status. Markers on graph indicate mutant Alanine samples

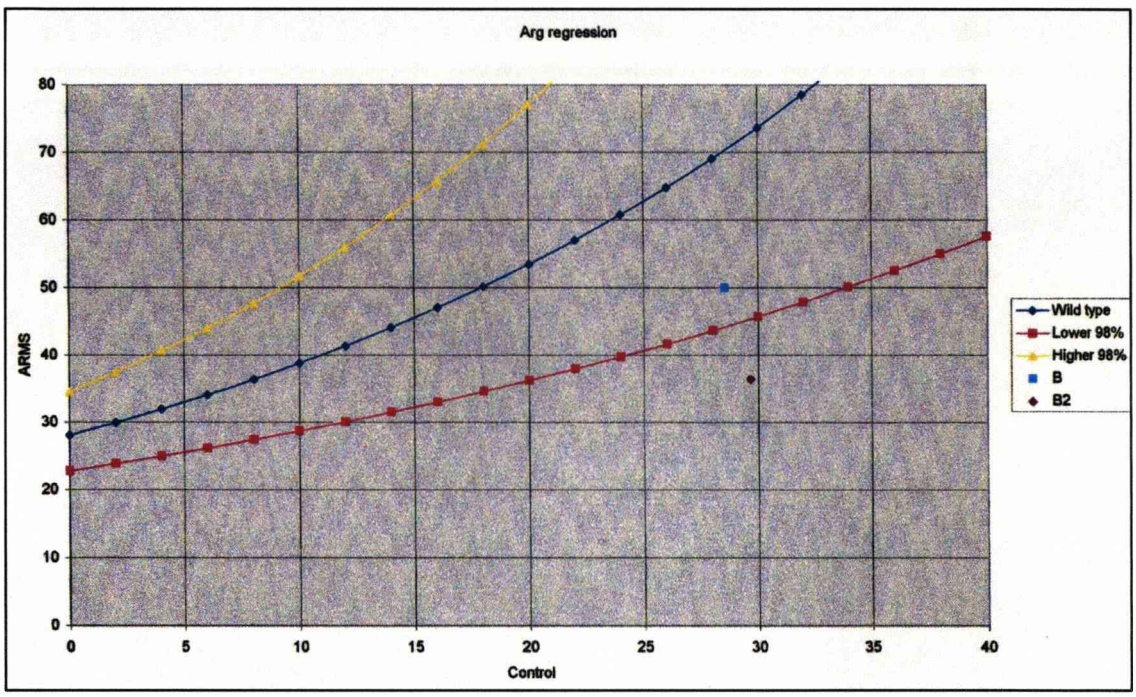


Figure 48: Graph to determine the Kras Arganine mutation status. Blue and purple markers on the graph indicate mutant Arganine samples

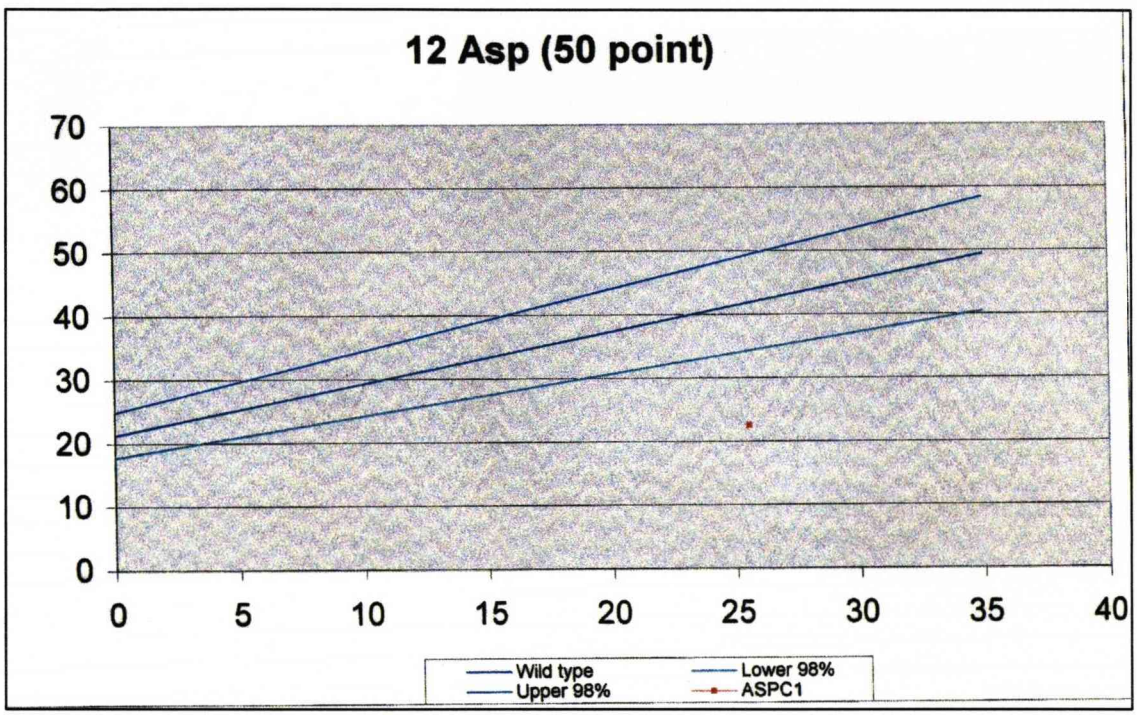


Figure 49: Graph to determine the Kras 12 Aspartate mutation status. Shaped markers on graph indicate mutant 12 Aspartate samples

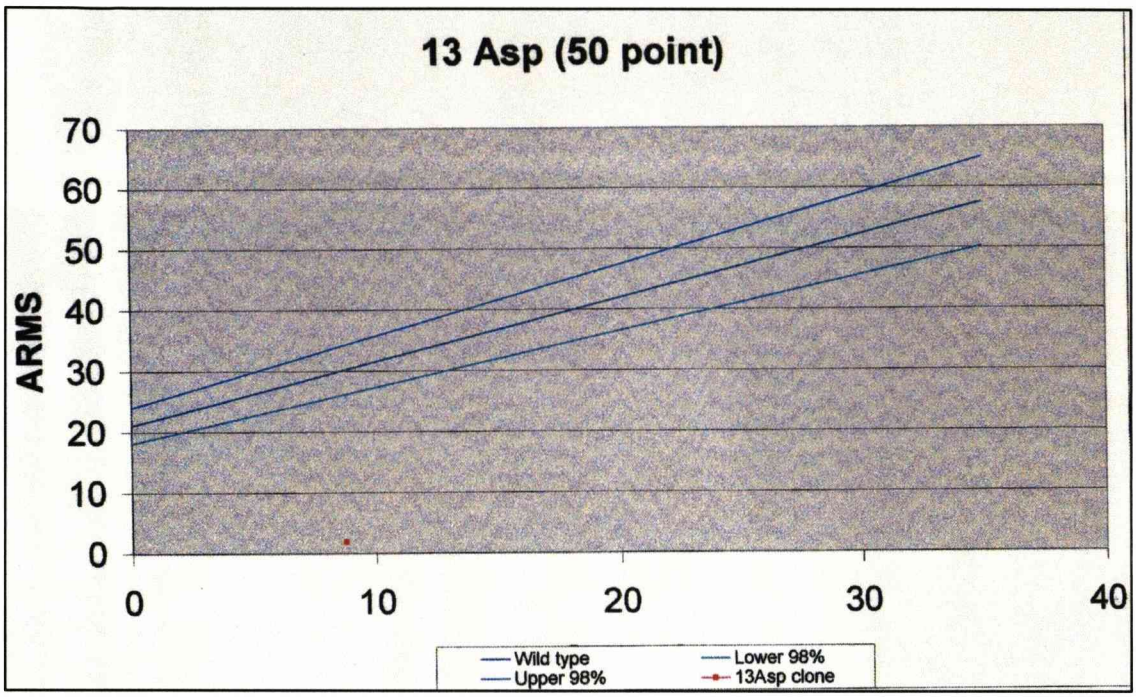


Figure 50: Graph to determine the Kras 13 Aspartate mutation status. Shaped markers on graph indicate mutant 13 Aspartate samples

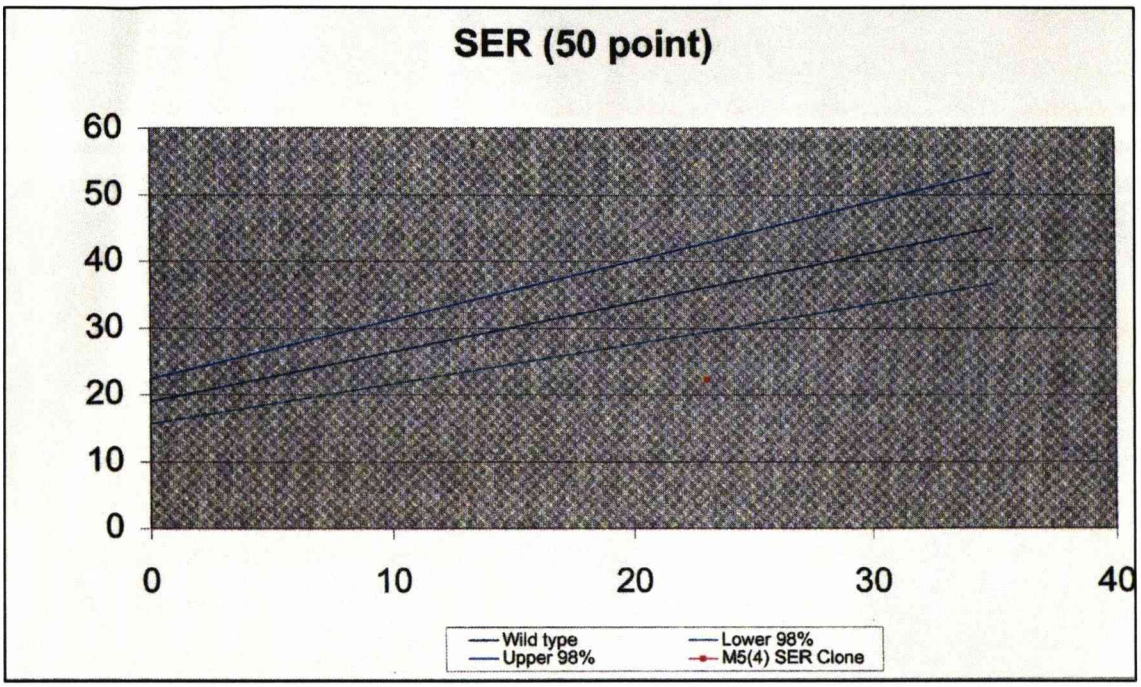


Figure 51: Graph to determine the Kras Serine mutation status. Shaped markers on graph indicate mutant Serine samples

Statistics for Kras Mutations Analysis

Statistical analysis was carried out using Statview and Microsoft Office Excel 2003. Excel worksheets, sensitivity and specificity equations were used for Kras mutational analysis in pancreatic juice, bile, tissue in pancreatic ductal adenocarcinoma, ampullary cancer, chronic pancreatitis and control patients. Also, analysed were matched tissue samples with pancreatic juice and bile for Kras mutations.

Excel worksheets were also used to generate the age distribution Kras mutation graphs in the samples in the various patient groups. Arbitary scale graphs for Kras concentration quantification in bile and pancreatic juice were produced using Microsoft Excel.

Statview was used to analyse the distribution of Kras with age using the Mann-Whitney U, Chi-square and Logrank tests. Kras mutation based on disease and type of mutation was analysed using the Logrank tests with Statview.

Results

Demographics for Kras Mutations Analysis

Overall, 276 patients with pancreatic ductal adenocarcinoma, chronic pancreatitis, ampullary cancer and control (benign biliary diseases) were recruited. Seventy five pancreatic juice, 142 bile and 59 tissue samples were analysed. The median age of patients for pancreatic ductal adenocarcinoma was 63 years, for chronic pancreatitis was 54 years and control was 73 years.

Sample/ Disease	Pancreatic Juice	Bile	Combination	Tissue
PDAC	17/26 (65%)	12/28 (43%)	27/50 (54%)	20/29 (68%)
Ca. Amp	6/7 (86%)	6/9 (67%)	10/14 (71%)	7/17 (41%)
C. P.	10/30(33%)	4/15 (27%)	12/40 (30%)	10/13 (31%)
Control	5/22 (23%)	26/90 (29%)	19/105 (28%)	N/A
Sensitivity	70%	49%	58%	58%
Specificity	71%	71%	72%	69%

PDAC= pancreatic ductal adenocarcinoma, Ca. Amp= Ampullary cancer, C. P. = Chronic Pancreatitis.

Table 31: K-Ras Mutations Analysis

ARMS analysis of Kras mutations in pancreatic juice of patients with PDAC, Ampullary Cancer, Chronic Pancreatitis and Control patients (i.e. patients with benign biliary diseases) showed Kras mutations to be present in 17/26 (65%) of patients with PDAC, 6/7 (86%) patients with ampullay cancer, 10/30 (33%) patients with chronic pancreatits and 5/22 (23%) control patients. These results show a positive trend that patients with malignancies have a higher rate of mutant Kras compared to patients with benign diseases (sensitivity 70%, specificity 71%).

Analysis of bile for Kras mutations show a similar trend- higher presence of Kras mutations detected in patient samples with malignancy compared to non-malignant patient samples. The results for the bile analysis are as follows- 12/28 (43%) of patients with PDAC, 10/14 (67%) patients with ampullay cancer, 12/40 (30%) patients with chronic pancreatits and 19/105 (28%) control patients (sensitivity 58%, specificity 71%).

We also analysed tissue samples from patients and found the presence of Kras mutations in 20/29 (68%) of patients with PDAC, 7/17 (41%) of patients with ampullary cancer and 10/13 (31%) of patients with chronic pancreatitis.

	Tissue K-Ras	Pancreatic Juice	Bile	Combination
PDAC	**Mutant**	7/9 (78%)	5/8 (63%)	10/14 (72%)
	Wild type	2/9 (22%)	1/2 (50%)	2/7 (29%)
Ca. Amp	**Mutant**	3/3 (100%)	3/3 (100%)	5/5 (100%)
	Wild type	0/2 (0%)	2/3 (67%)	2/5 (40%)
C. P.	**Mutant**	4/7 (57%)	2/2 (100%)	4/7 (57%)
	Wild type	6/9 (67%)	3/5 (60%)	9/14 (65%)
Sensitivity		73%	77%	73%
Specificity		40%	60%	50%

Table 32: Matched Samples K-Ras Mutations Analysis

We next analysed matched tissue/pancreatic juice and tissue/bile samples to determine the correlation of tissue K-Ras status with pancreatic juice/bile K-Ras status.

In patients with PDAC, 18 patients had matched tissue/pancreatic juice samples and 10 patients had matched tissue/bile samples. In 9 K-Ras mutant tissues, 7/9 (78%) pancreatic juice samples were K-Ras mutant. In 9 K-Ras wild-type tissues, 2/9 (22%) pancreatic juice samples were K-Ras mutant. In 8 K-Ras mutant tissues, 5/8 (63%) bile samples were K-Ras mutant and in 2 K-Ras wild-type tissues, 1/2 (50%) bile sample was K-Ras mutant.

In the ampullary cancer group, there were 5 matched tissue/pancreatic juice samples and 6 matched tissue/bile samples. 3 patients with K-Ras mutant tissues had corresponding K-Ras mutant pancreatic juice, 2 patients with K-ras wild type tissues had K-Ras wild type pancreatic juice. 3 patients with K-Ras mutant tissues had K-Ras mutant bile samples and in 3 patients with K-Ras wild type tissues, 2 (67%) had K-Ras mutant bile samples.

In the chronic pancreatitis patients, there were 16 matched tissue/pancreatic juice patient samples and 7 matched tissue/bile patient samples. For the 7 K-Ras mutant chronic pancreatitis tissues, 4 (57%) pancreatic juice were K-Ras mutant. For the 9 K-Ras wild type tissue samples, 6 (67%) were K-Ras mutant. In the matched tissue/bile group, 2 patients with K-ras mutant tissues also had K-Ras mutant bile (100%) and of the 5 K-Ras wild type tissue samples, 3 (3/5; 60%) had K-Ras mutant status.

	"Gold Standard" K-Ras	Tissue	Pancreatic Juice and Bile
RFLP	**Mutant**	4/4 (100%)	4/4 (100%)
		2 PDAC, 2 Ca. Amp	
	Wild type	6/10 (60%)	6/11 (55%)
		3/3 PDAC, 0/2 Ca. Amp, 3/4 Other, 0/2 C. P.	
LCM and Sequence	**Mutant**	6/10 (60%)	6/11 (55%)
		2/2 Ca. Amp,3/4 PDAC, 0/3 Other, 1/1 C. P.	
	Wild type	2/4 (50%)	1/4 (25%)
		1/2 Ca. Amp, 1/2 C. P.	

RFLP= restriction fragment length PCR, LCM= Laser Capture Microscopy

Table 33: Comparison of ARMS and other techniques

The RFLP and LCM and sequencing techniques are considered the 'Gold Standard' for detecting K-Ras mutations and the Table above presents our results from analysis of clinical samples of relevance.

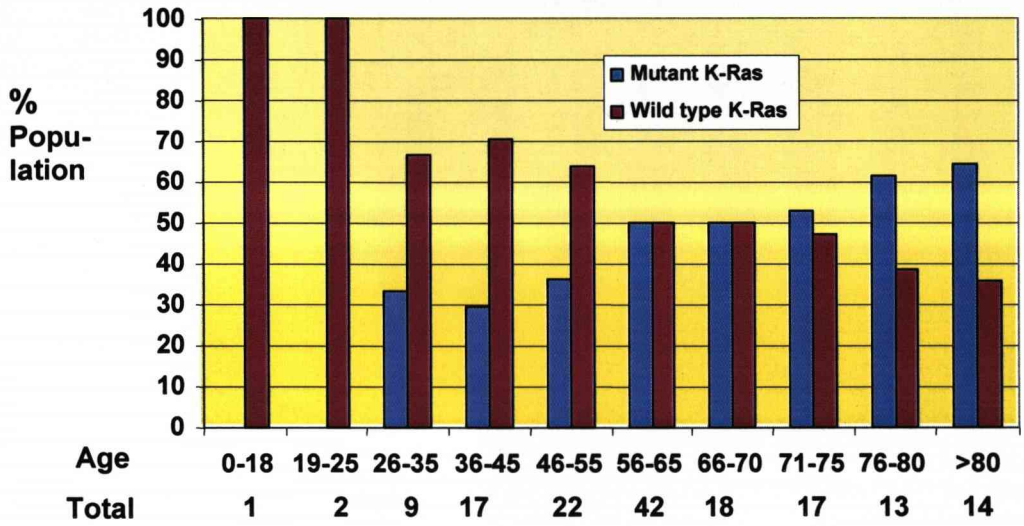


Figure 52: Age Distribution of K-Ras Mutation in All Samples Analysed

The analysis of the presence or absence of K-Ras mutations by age across all patient samples (PDAC, chronic pancreatitis and control patients) revealed a trend of increasing presence of K-Ras mutation with increasing age. In our patients sample analysed there were no K-Ras mutations detected in patients less that 25 years old.

	Mutant Ras	Wild type Ras	Significance (p value)	
			M-W U	Logrank
Total	64 (IQR: 57-74)	57 (IQR: 44-68)	0.003	0.046
Controls	73 (IQR: 61-80)	64 (IQR: 49-70)	0.056	0.027
C. P.	54 (IQR: 46-64)	46 (IQR: 39-55)	0.103	0.083
PDAC	63 (IQR: 59-73)	63 (IQR: 48-74)	0.809	0.449

Table 34: Distribution of K-Ras Mutation with Age Median Age

The table above demonstrated the median age of patient with mutant and wild type K-Ras in the different groups anaylsed. The median age for the Control, chronic pancreatitis and PDAC groups are 73 years, 54 years and 63 years respectively.

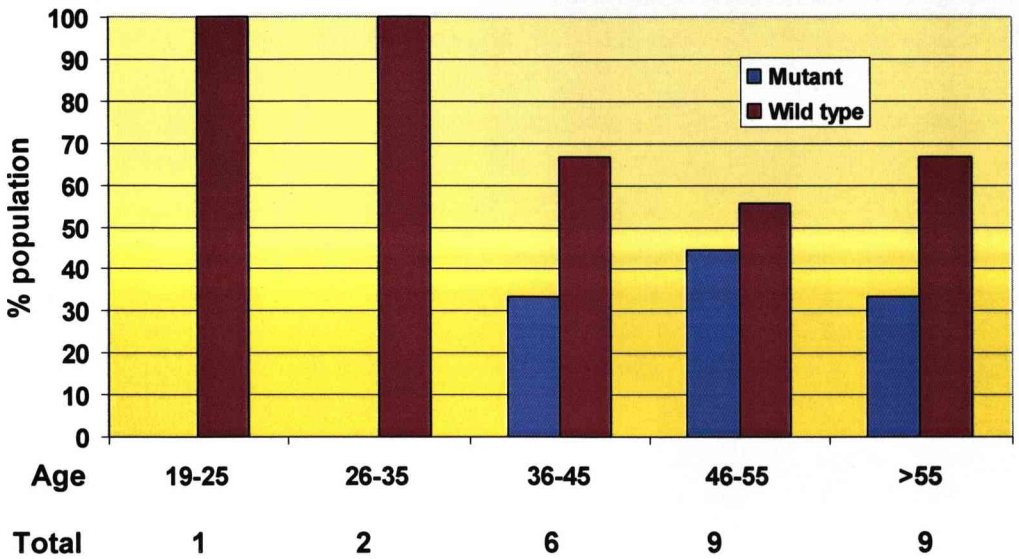


Figure 53: Age Distribution of K-Ras Mutation in Chronic Pancreatitis Samples Analysed

Analysis of K-Ras mutations by age and diagnosis- in this case, patients with chronic pancreatitis, showed that there were no K-Ras mutations in patients under the age of 35 years old. In our patient group, K-Ras mutations appeared most prevalent in the 46-55 years age group.

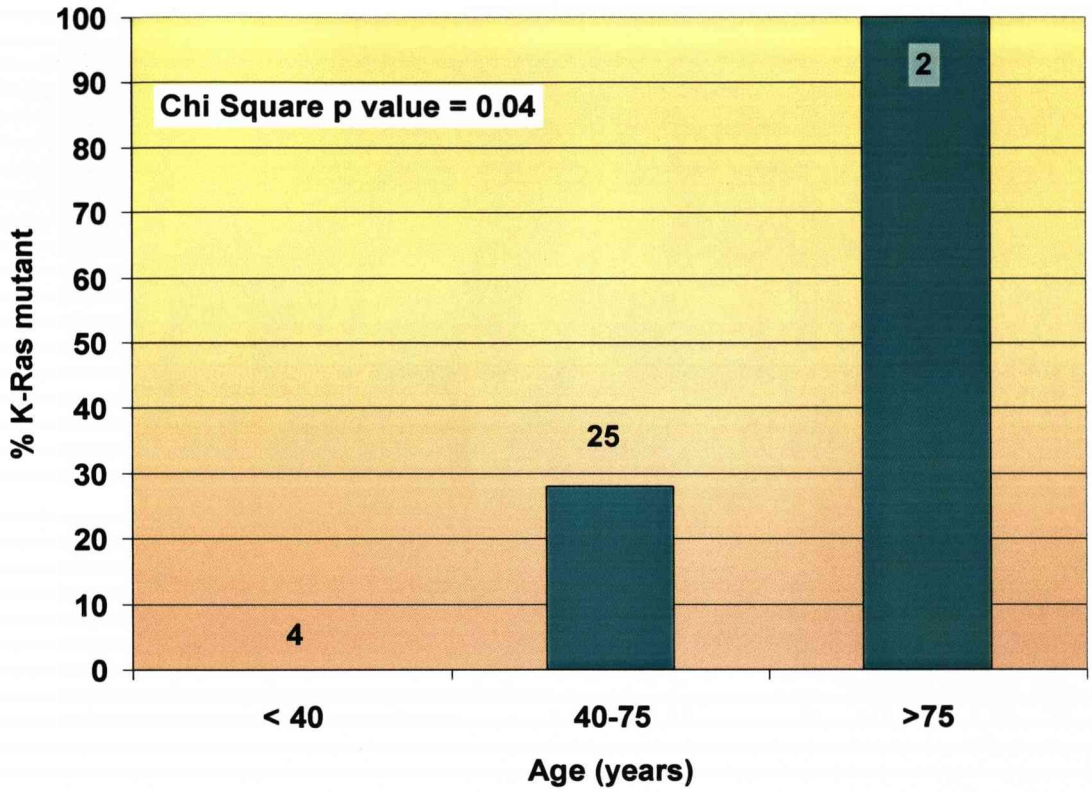


Figure 54: Correlation between age and K-Ras mutation in Chronic Pancreatitis patients

With a slightly larger patient chronic pancreatitis group, again it is shown that K-Ras mutant is not present in patients under the age of 40 years and occur more frequently with advancing age.

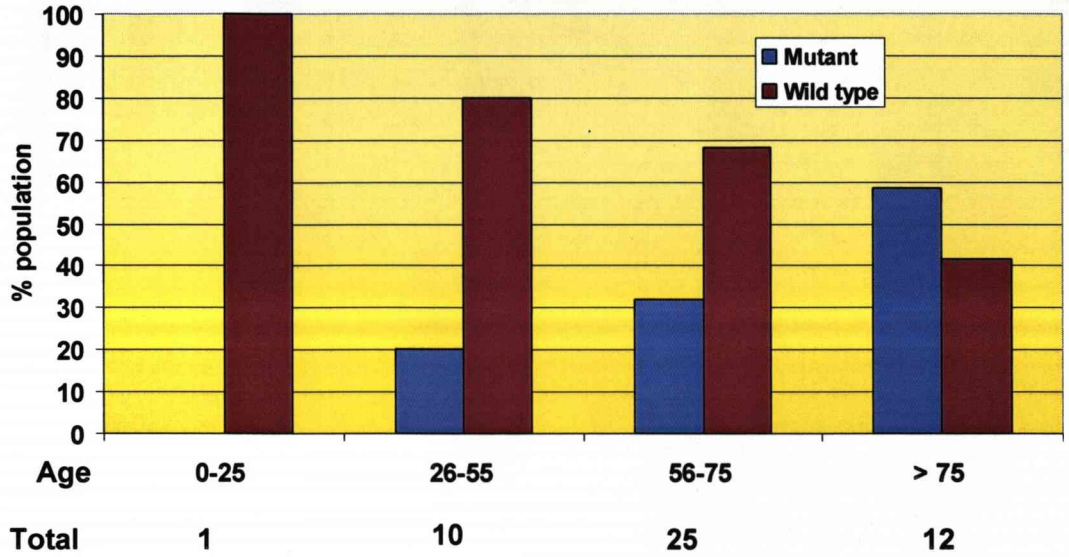


Figure 55: Age Distribution of K-Ras Mutation in Control Samples Analysed

In the Control patient group, there was a positive trend for K-Ras mutation with increasing age. There was no K-Ras mutation in the under 26 years old group.

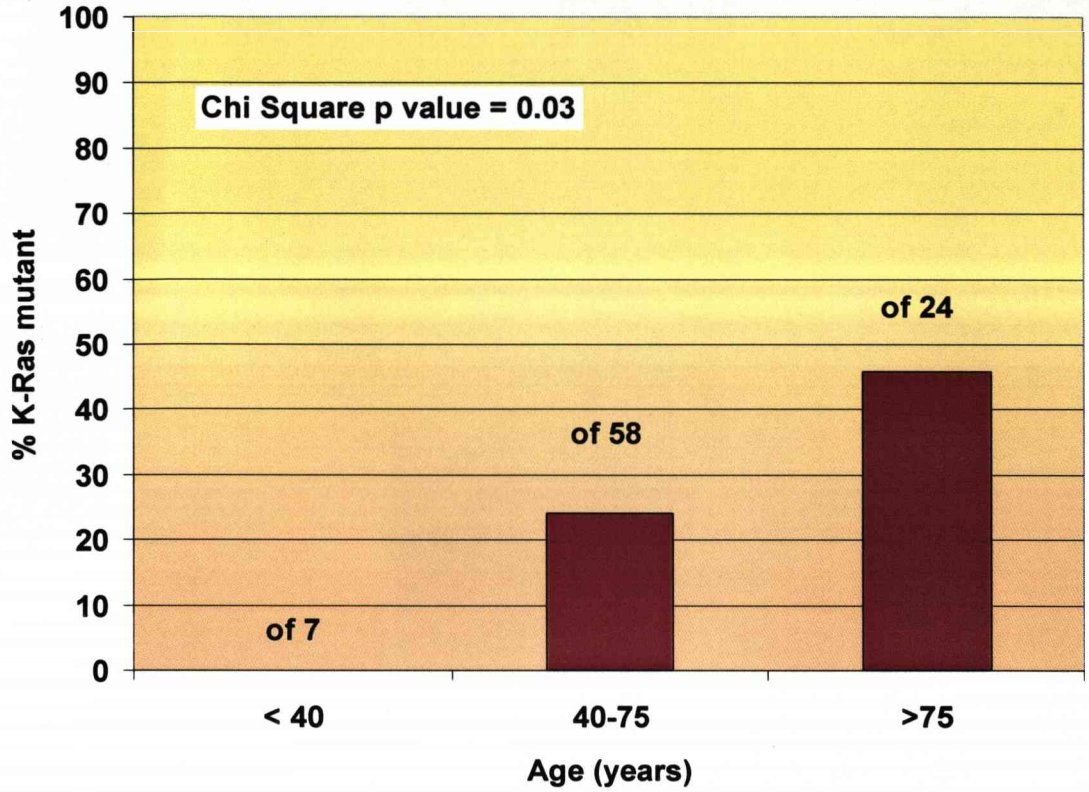


Figure 56: Correlation between age and K-Ras mutation in Control patients

The figure above demonstrated the stastistically significant positive trend for the presence of K-Ras mutation with increasing age in Control patients.

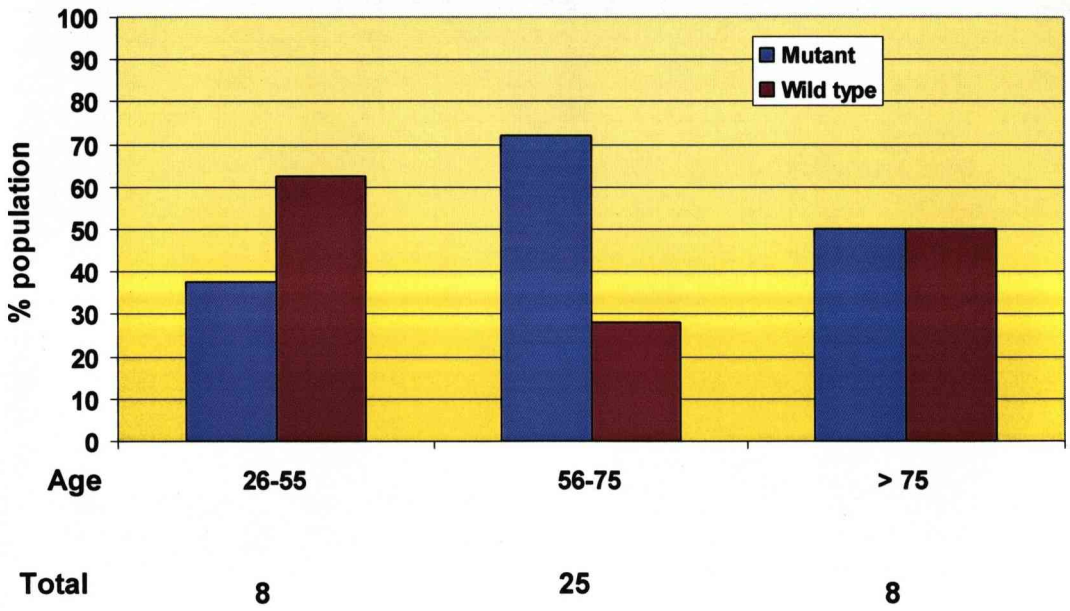


Figure 57: Age Distribution of K-Ras Mutation in PDAC Samples Analysed

Lastly, in the PDAC patients, the presence of K-Ras mutation was seen mainly in the 56-75 years age bracket. K-Ras mutation was also observen in the younger 26-55 years age group.

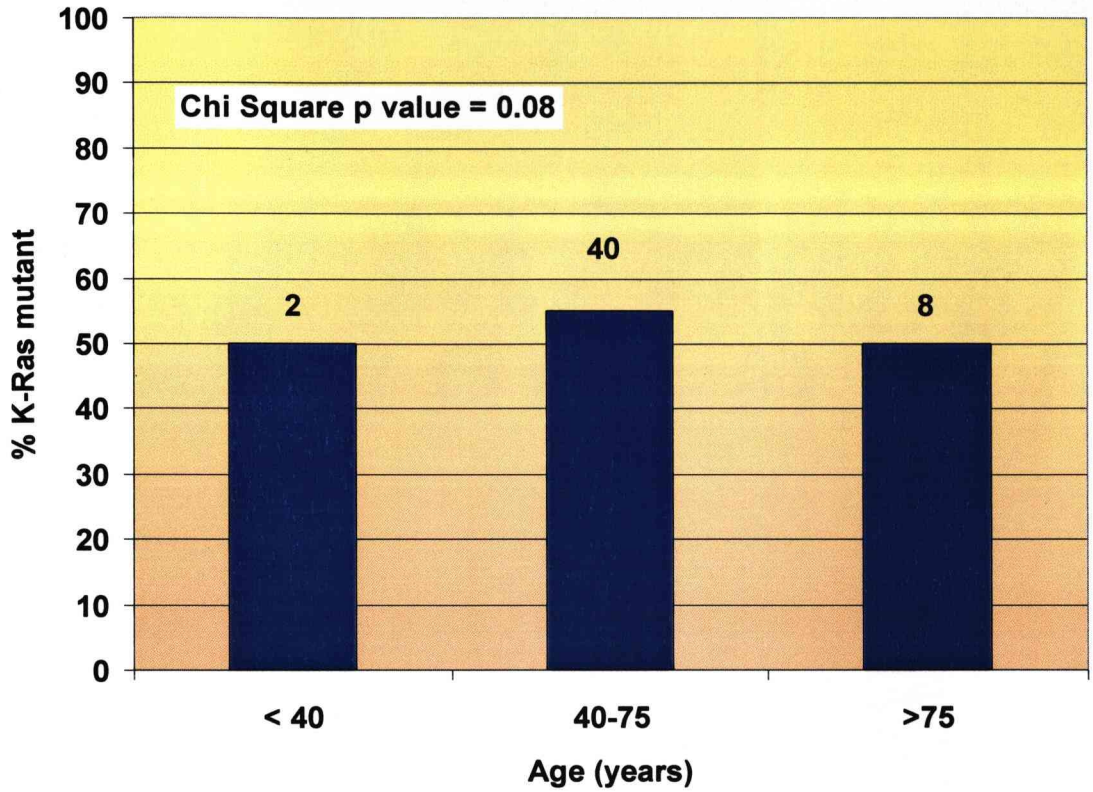


Figure 58: Correlation between age and K-Ras mutation in PDAC patients

In the PDAC patient group, K-Ras mutation occurs in almost equal frequencies across all the age groups.

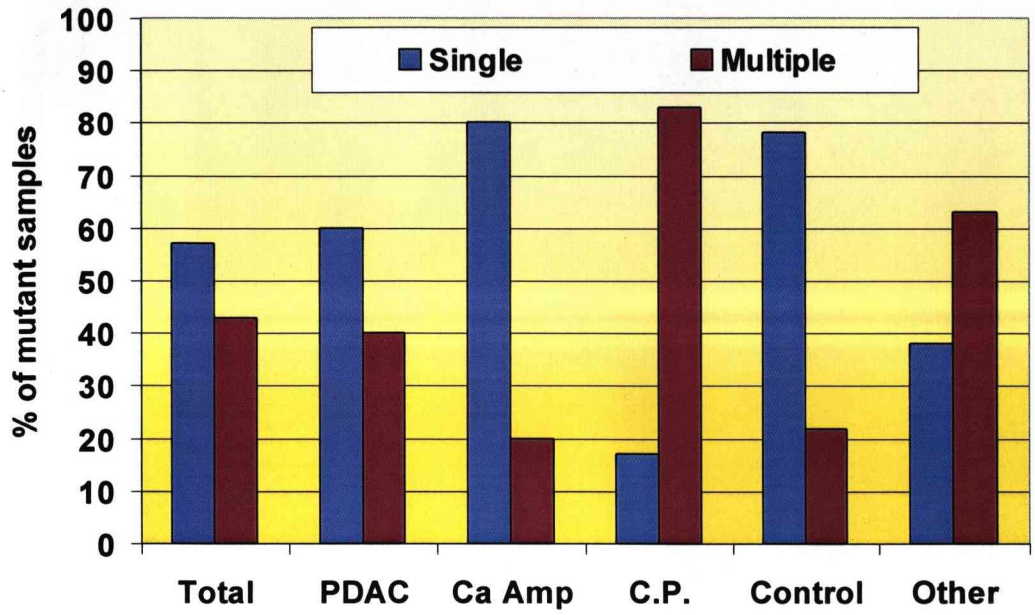


Figure 59: Analysis of K-Ras Mutations based on Disease and Number of Mutations

When we looked at the relationship between disease diagnosis and the number of K-Ras mutations present in pancreatic juice/bile samples analysed (as shown in the figure above), we found that in the patient groups PDAC, Ampullary cancer and Control- there were one K-Ras mutation present in 60%, 80% and 79% of the samples analysed respectively.. In the chronic pancreatitis group, multiple K-Ras muations was detected in 82% of the samples.

	Pancreatic Juice	Bile	Combination
PDAC	10/22 (45%)	8/23 (35%)	15/41 (37%)
Ca. Amp	5/6 (83%)	4/9 (45%)	8/13 (62%)
C. P.	2/27 (7%)	1/13 (8%)	2/36 (5%)
Control	4/9 (44%)	10/43 (23%)	14/49 (29%)
Sensitivity	54%	37%	45%
Specificity	83%	81%	81%
Specificity PDAC/ C. P.	93%	92%	95%

Table 35: Distribution of Single K-Ras Mutations in Pancreatic Juice and Bile

In this table, again it show that in the diagnosis for PDAC, ampullary cancer and control the occurance of single K-Ras mutations occur at a frequency of 45%, 83% and 44%. However, when combined with the results from the K-Ras analysis of bile, this figure is skewed to a lower level.

In the chronic pancreatitis group, the results show consistently the high prevalence of multiple K-Ras mutations.

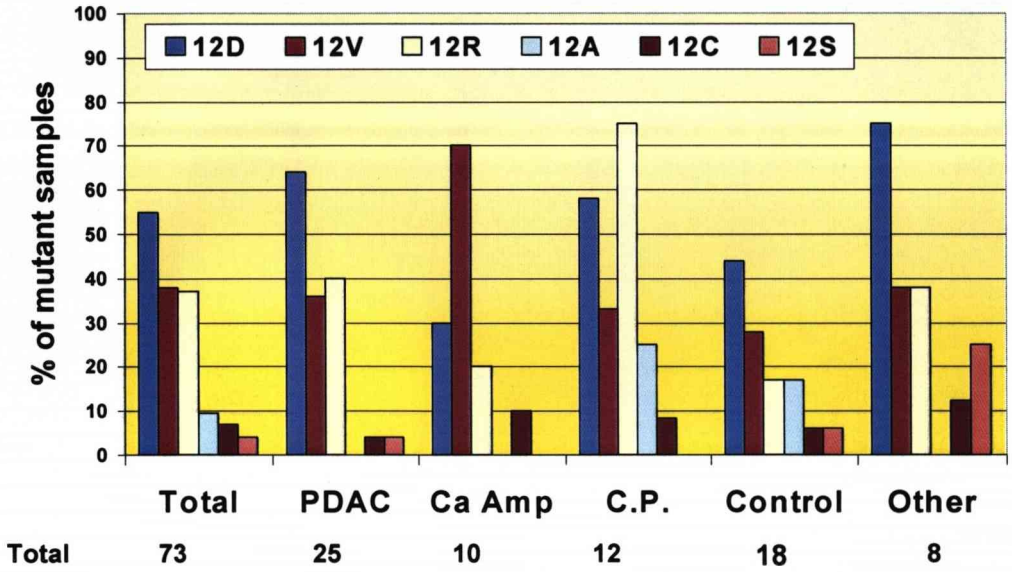


Figure 60: Analysis of K-Ras Mutations based on Disease and Type of Mutations

In the PDAC group, there were a high proportion of K-Ras 12 Aspartate mutations, followed by Arganine, Valine and a small number of Cysteine and Serine mutations. In the Ampullary cancer group, the most prevalent K-Ras mutation was the Valine mutation followed by the 12 Aspartate, Arganine and Cysteine mutations. In both the PDAC and Ampullary cancer groups analysed, the Alanine K-Ras mutation is not present.

In the chronic pancreatitis group, there are high levels of the Arganine and 12 Aspartate K-Ras mutations followed by lower levels of Valine, Alanine and Cysteine mutations. The 12 Aspartate is again the most prevalent mutation in the Control group.

Overall, the Serine K-Ras mutation occurs at low levels across the diagnosis groups.

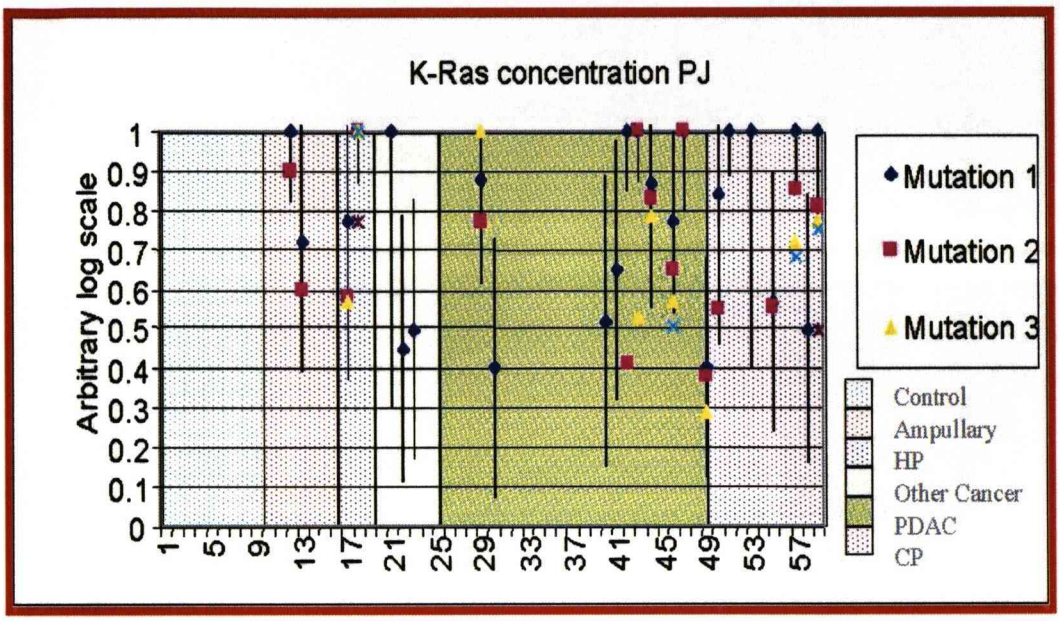


Figure 61: Quantification: Concentration of K-Ras in Pancreatic Juice

Arbitary quantification of K-Ras concentration in pancreatic juice in the different patient diagnosis groups show that generally, the concentration of mutant K-Ras is equivalent in all the groups.

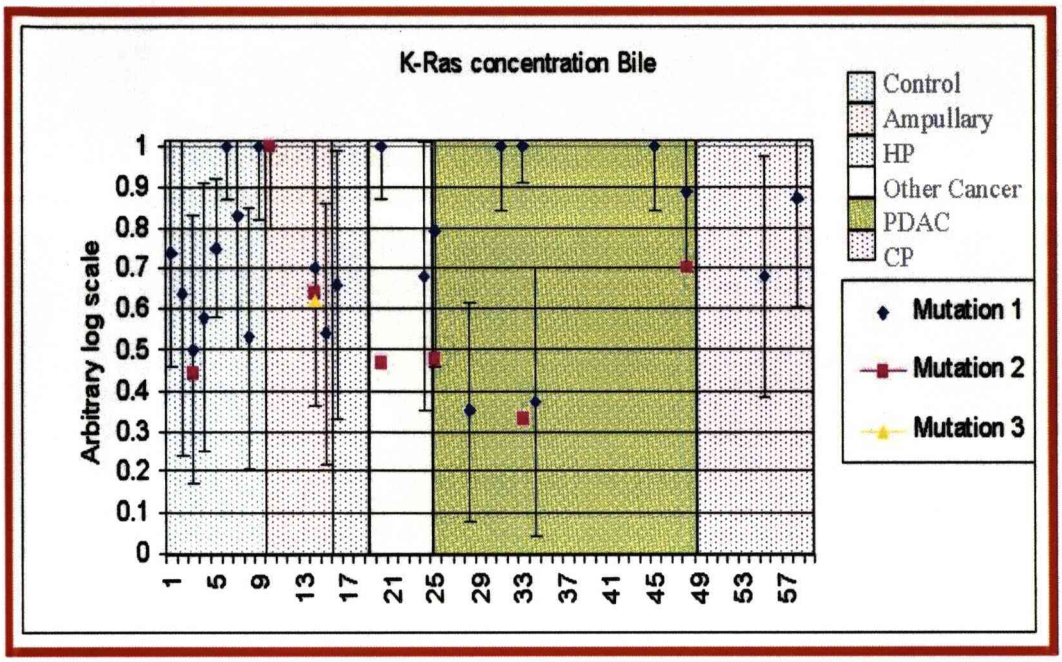


Figure 62: Quantification: Concentration of K-Ras in Bile

The K-Ras mutations concentration bile are roughly equivalent to that in pancreatic juice, although in the PDAC group where there is greater variation in the concentrations present.

Discussion

Premalignant molecular changes can be detected in the pancreatic juice of patients. Thus the application of molecular screening in patients with HP is potentially the most powerful method of pancreatic early cancer detection. Although mutant K-ras can be detected in the pancreatic juice of most patients with pancreatic cancer, it is also present in patients with non-inherited chronic pancreatitis who do not progress to pancreatic cancer (at least in the short- to medium-term) as well as increasingly in the older population without pancreatic disease as described in this thesis. Nevertheless the presence of mutant K-ras may identify a genuinely higher risk group enabling additional diagnostic imaging and molecular resources to be focussed onto such a group.

Chapter 5- Conclusion

Exon 4 Polymorphism PRSS1

For both R122H and N29I there is no strong evidence for a single recent founder. For patients with no PRSS1 mutation there is no evidence for a link to PRSS1.

There no evidence that the R122H and N29I hereditary pancreatitis families come from a single population (defined by PRSS1). The proportion of the mutation linked to the low frequency allele was 30% and 75%. The expected frequency from the control population would be between 36 and 46%. So the number of families with mutations linked to the low frequency allele is somewhat higher than expected for N29I and somewhat lower for R122H, but there are clearly 2 founder populations for each mutation type.

If an as yet unidentified mutation in PRSS1 is responsible for the disease in HP patients with none of the established mutations then we would again expect a disproportionate number of families with either the low frequency or the high frequency allele linked to the disease. In fact 21% of families had association with the low frequency allele, slightly lower than expected (36 to 46%) but certainly not consistent with a single common founder having a mutation on PRSS1.

As mentioned previously, the D32D polymorphism had been observed by Nishimori et al when analysing the cationic trypsinogen gene in Japanese families with HP

(Nishimori et al. 1999). This group also reported a polymorphism (a single C to T transition without amino acid substitution) in exon 5 in some patients with HP, sporadic pancreatitis and in normal subjects. Gorry et al also reported a normal polymorphism in exon 4 of the PRSSI gene (Gorry et al. 1997).

Future work

During the time of the study, the available samples limited the power and analysis of the study. The incidence of this polymorphism is worth further investigation in order to establish any possible founder effect. This is important because cancer risk is almost certainly influenced by the broader genetic background. Thus it is likely that some families have more risk of cancer than others and so different founder populations should be screened differently.

Conceptually the simplest way to investigate a founder effect is to simply increase numbers and with the current much larger database of patients available this seems a very attractive approach. However, power calculations put this study in perspective. If we assume a null hypothesis that our incidence of low and high frequency alleles in R122H and N29I are equivalent to controls (36-46%) and the hypothesis that the percentages seen in this preliminary study are as defined then how many controls and families would need to be tested? Let us assume we could test 500 controls this would mean that to show that R122H has significantly less low frequency allele carriers we would need to test 470 families (this is still beyond the scope of the EUROPAC registry.

In contrast only 20 N29I families would be needed, this is feasible and should be attempted.

It is perhaps of more academic interest to determine if there is any founder effect in those families with no PRSS1 mutation. Given the same assumptions as above we could prove a founder effect with just 50 negative for all families. This would mean that further work could be focused on finding the elusive additional PRSS1 mutation, focussing on the families with the high frequency allele.

An alternative approach to studying founders in all HP families would be the use of SNP array analysis, this could be used in an association study to identify other loci that cause or influence outcome in HP. Again the biggest problem would be power. This is more difficult to calculate given that we know little about relative allele frequencies in the different populations, but it is unlikely to require fewer families than with the more specific analysis of the PRSS1 locus.

In all the analyses above a correlation between the polymorphisms and cancer risk would have to be established, this will require survival analysis in the different groups.

P16 and MGMT methylation

In this study, we looked at the methylation status of DNA found in pancreatic juice as indication about the diagnosis status and age of the patient. Free DNA can be detected in different body fluids- urine, synovial fluid, pancreatic duct secretions, sputum and serum/plasma. This circulating DNA in serum/plasma is found in small amounts in healthy individuals, but in cancer patients, higher concentrations of DNA are present. The serum of cancer patients is enriched in DNA, containing on average ~4 times the amount of free DNA, compared to normal controls (Shapiro et al. 1983; Wong et al. 2000). p53 and ras gene mutations are detected in plasma and serum of pancreatic and colorectal carcinomas (Sorenson et al. 1994; Mulcahy et al. 1998). However, in previous work in our laboratory no mutations could be detected in the plasma or serum of patients with respectable pancreatic cancer (Howes 2003).

Similarly, aberrant promoter methylation has been detected in serum of patients with malignancies. Estellar et al found correlation of DNA methylation status in serum and tissue samples in non-small cell lung carcinoma primary tumours. Promoter hypermethylation in serum DNA was 33% (3 of 9 cases) for p16 and 66% (4 of 6 cases) for MGMT. Two important points to note in this study are 1. The patients who have abnormal promoter hypermethylation in the serum DNA demonstrated identical alterations in the primary tumour DNA (this is not within the scope of this study) and 2. Only patients whose tumours harboured a hypermethylated marker showed aberrant methylation of the same genes in serum. In essence, this study showed, for the first time, that it was possible to detect promoter hypermethylation in serum DNA from cancer

patients. Ramirez et al found found good correlation between tissue and serum MGMT and p16 methylation in patients with glioblastoma (MGMT tissue 38.1%, MGMT serum 39.3%, p16 tissue 66.7%, and p16 serum 53.6%) (Ramirez et al. 2003). In light of our previous work where tumour DNA could not be detected in the plasma or serum of patients with resectable tumours we decided to concentrate our efforts at methylation analysis on pancreatic juice samples, which have a far more intimate contact with tumour tissue (Howes 2003).

The results for methylation in serum are supported by my study in as much as hypermethylation of p16 and MGMT were detectable in the pancreatic juice samples for methylation status analysis from our cohort of patients and can be reliably used as a technique for the molecular diagnosis of pancreatic cancer.

Recently, Fukushima et al reported his findings of methylated p16 DNA in pancreatic juice (Fukushima et al. 2003). Using methylation specific PCR, methylated p16 were detected in 11.1% (5/45) of pancreatic juice and in 18.2% (6 of 33) of tissue samples analysed in patients with primary pancreatic adenocarcinoma. In contrast, no methylated p16 DNA was detected in the pancreatic juice samples of the benign patient group (20 patients, including 12 chronic pancreatitis patients). Interestingly, pancreatic juice samples obtained from the duodenum of patients without pancreatic cancer were positive for methylated p16 in 11.1%.

From our analysis of pancreatic juice, we found 100% (30/30) p16 and 87% (26/30) MGMT methylation in patients diagnosed with pancreatric ductal adenocarcinoma (compared to 18.2% p16 methylation by Fukushima et al). In our benign disease group (combining the control and chronic pancreatitis patients), 48% (31/65) and 45% (27/60) patients had p16 and MGMT methylation respectively. The only variable not taken into account in our study is whether the pancreatic juice were obtained from the pancreatic duct or the ampulla of Vater, as it has been shown by Fukushima et al, that the site of pancreatic juice sample collection could influence the methylation status of samples. If the findings can be reproducible, that is, pancreatic juice from patients with benign diseases does not contain methylated p16 or MGTM, then it may be possible to detect early PDAC from the methylation status of p16 and MGMT from pancreatic juice obtained from the pancreatic duct.

Future work

In subsequent work by my colleague Dr Li Yan methylation in control samples was reported, but the level was much lower than in the pancreatic juice from cancer patient (Yan et al. 2005).

Current work is being undertaken to determine whether DNA methylation from pancreatic juice can be validated and used as an effective biomarker for the detection for pancreatic cancer.

According to previous results, using quantitative *p16* promoter methylation together with *p53* and *K-ras* mutation in DNA from pancreatic juice could distinguish the control individuals from those suffering from chronic pancreatitis or cancer. This combination seems to be useful to assess cancer risk in high risk individuals.

In the current study ten tumor-suppressor, growth regulatory or mismatch repair-related genes were screened for aberrant methylation in pancreatic cancer, including *p16* (tumor-suppressor gene), *hMLH1* (Mut-L homologue 1, involved in mismatch repair and associated with Hereditary Non-Polyposis Colorectal Cancer), *MGMT* (O-6-methylguanine-DNA methyltransferase, removes adducts from the O6 position of guanine), *MINT2, MINT31, MINT32, ppENK* (preproenkephalin, encoding for a native opioid peptide with growth-suppressor properties), *FOXE1, TFPI2, NPTX2*. Quantitative methylation analyses are being performed on pancreatic juice DNA.

Pancreatic Cancer and K-Ras

It is essential that the test used to identify mt-K-ras is highly sensitive and specific. We have developed the application of ARMS™ technology, which is sensitive enough to detect one mutant copy of DNA in 5000 copies of wild type and has the additional advantage of being quantitative for mutant DNA in the sample, without reducing the overall sensitivity of the technique (Wong et al. 2000). We are unaware of better technology in place and being used in a clinical setting (Howes et al. 2000).

What is clear is that prospective multi-centre studies, such as that being pursued by EUROPAC, is essential for the development of an effective secondary screening programme for these patients. Very early cancers (<1cm in diameter and no lymph node metastases) may result in five-year survival rates of ~50% and the prospect of genuine cure – unfortunately such tumours are rarely found in routine clinical practice except by serendipity (Tsuchiya et al. 1986). Nevertheless this observation shows that a meaningful proportion of patients might be cured if the tumours could be detected early enough. Thus the question arises as to the possibility of primary screening of the healthy population.

Future work

Unfortunately, K-ras mutations are not specific for invasive pancreatic cancer. Previous investigations on K-ras mutations in pancreatic tissue, pancreatic juice and stool revealed its occurrence in patients with chronic pancreatitis, in individuals who smoke, and in PanINs from patients without pancreatic cancer. To increase the power, sensitivity and specificity of the studies, large numbers of patient samples needs to be analysed especially with regards to paired samples for correlation between Kras mutations found in blood/ pancreatic juice/ bile and diseased tissue samples.

During the interim of this thesis being prepared and submitted, the technique developed has been used in research studies at the Department of Surgery and Oncology, University of Liverpool, for the analysis of K-ras mutations in patients identified as

high-risk. The technique is part of a 3 prong approach in the risk stratification of cancer development.

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